

nature

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No error-free society

NOTHING ventured, nothing gained—the calculus of risk and the trade-off between benefit and damage is much in the news at present. This is partly due to the growing public interest in decision-making in the nuclear industry, but some of the credit in Britain must go to the Council for Science and Society (CSS) which has had a study group looking at the question of acceptable risk and which this week convened a public meeting on the subject.

There is something almost irrational in our variable attitudes to risk-taking. The hazards of cigarette smoking are well enough known, are not by any means negligible, often involve others quite involuntarily in a long period of distress and yet are clearly deemed acceptable by a large section of the public. The same might be said of combining drinking with driving. And yet at the other end of the scale those same inveterate risk-takers may show profound shock on hearing of ten people being killed in a coach crash or may express the strongest opposition to the building of a nuclear power station with a claimed minute chance of having a serious accident. We may live in fear of a major accident in which a thousand might die even though, as Reg Farmer pointed out last week (page 93), such an event would barely affect the accidental death rate. It somehow seems that the idea of one or two people being responsible for many deaths strikes a very profound note in the public conscience. Desirable as this is, it inevitably dampens down public concern for how to cut down on the smaller scale accidents.

A frequent rallying cry amongst those concerned with hazards is that as technology grows so does the risk. Even the CSS will say in its working party's report 'popular concern about risks has increased recently, as man-made disasters carry the threat of even worse to come from new or projected technologies'. A more technological world may indeed open up the possibility of human error causing more deaths; the six hundred deaths on Tenerife would have been only one hundred from a similar accident twenty-five years ago. But all in all the fatality rate for air travel has been steadily declining during this same period that the potential for bigger tragedies has been growing, so maybe the intro-

duction of new technology, some of which is safety-oriented, has had a beneficial effect.

Because things have improved in one well-studied industry doesn't, of course, mean the same will happen everywhere, and it is obviously right that we should go on asking more and more searching questions about safety. But modern scientific analysis coupled with intensive computer simulation of hazards can easily give the public a false impression that by better and deeper study the present-day technologist can give more and more assured guarantees that anything newly introduced will be 'safe'. As accidents from the trivial to the severe show, however, human actions, logical in themselves but illogical in a broader context, are at the heart of many disasters. These actions can never be properly simulated—who would have put a candle into the computer program for safety at Browns Ferry?

What we have to do if we really want the benefits of new technology is to be prepared for some mistakes to find their own way out—a principle, incidentally, followed throughout the whole process of scientific advance.

Obviously we don't allow a few stored nuclear weapons to go off by accident in order to learn how they can be safely stored, but in many environments, such as factories, air traffic, coal mining, novel engineering structures and so on, it is only with the help of an occasional accident, however distressing, that some very important and valuable pieces of information ever emerge. In an age in which the public expects scientists and technologists to think of everything ahead of time, this idea of learning from mistakes is often forgotten. But to learn effectively, the after-the-fact enquiry must be very wide ranging, because accidents, even road accidents, frequently result from a string of errors, sometimes apparently of little relevance. Thus one requirement, insufficiently widely discussed, in risk reduction is that the scope of enquiries should not be too severely circumscribed. This is a political matter and decisions are often taken against a background of fears of a 'fishing expedition'; maybe there is some good research to be done on how wide committees of enquiry should spread their nets. □



When is a drought a drought?

Michael H. Glantz and Richard W. Katz, of the Environmental and Societal Impacts Group at the National Center for Atmospheric Research, Boulder, Colorado, draw on the case of the Sahel to comment on the use of the term 'drought' for arid regions

THE word 'drought' has various interpretations: decision-makers, political and otherwise, underestimate its likelihood in and around regions where interannual rainfall variability is relatively high. In such arid and semiarid areas, recurrent droughts are in fact a part of climate and not apart from it, and therefore should not be viewed as unexpected events.

The West African Sahel, one such area, has recently been affected by its third major extended drought this century. Many observers have suggested that the drought, which began in various parts of the region in 1968, ended with a return to normal or near normal rainfall. Some interpreted the relatively good rains in 1974 as indicative of a resumption of 'normal' rainfall. Similar statements have been common in the growing volume of drought literature. However, they can mislead because they imply that a return to normal rainfall, that is, rainfall seen as an 'average', is associated with a return to acceptable if not favourable meteorological, agricultural and social conditions for the region. The validity of such an assumption is questionable.

What is perceived as 'normal' rainfall is an important, yet often overlooked, aspect of what is meant by 'drought' in arid and semiarid regions. People react to their perceptions whether or not those perceptions are based on reality, and the consequences of those reactions are real. It is misleading to view 'normal' as a 'normative quantity' with the implication that a departure is 'abnormal': a 'normal' has no predictive value. The literature concerned with droughts in arid and semiarid regions is replete with references to particularly dry years as being abnormal or anomalous. Yet, because perceptions about what con-

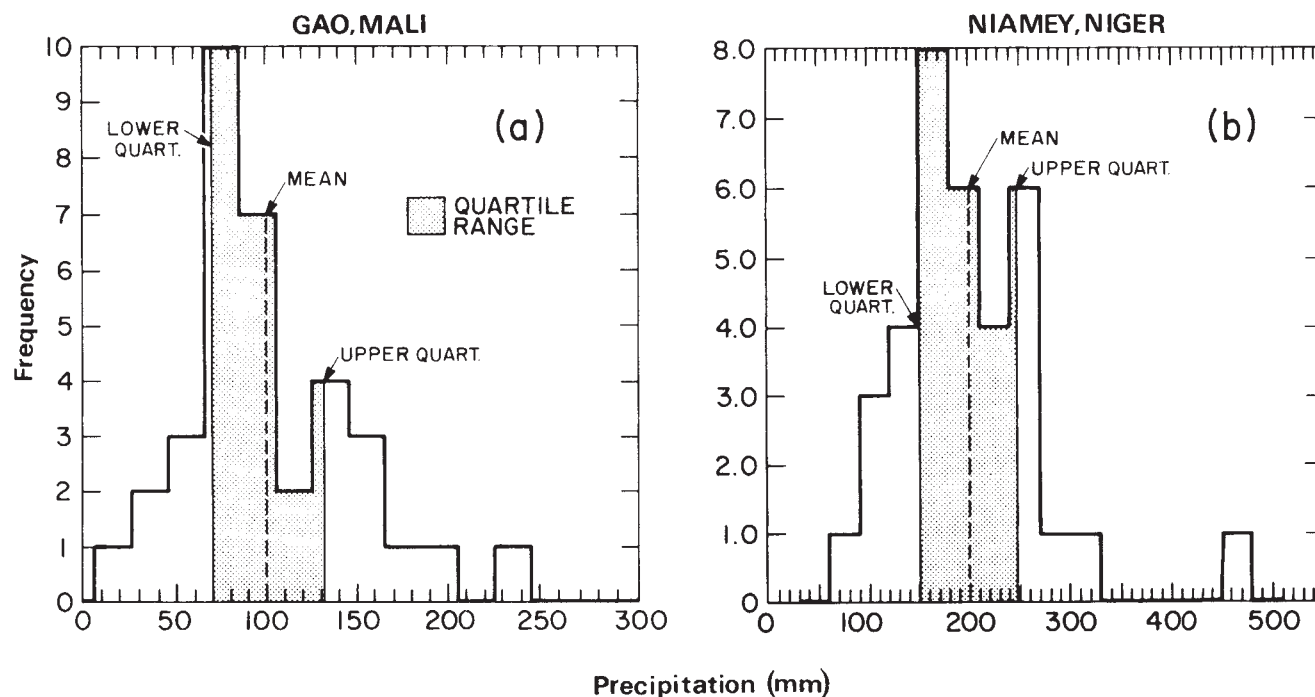
stitutes a drought vary, there is no widely accepted definition of drought. The scores of existing definitions, however, are either meteorological or agricultural. A meteorological drought could arbitrarily be defined as that period when the amount of precipitation is less than some designated percentage of the long-term mean, whereas an agricultural drought could be defined in terms of the timing of the rainfall which is crucial to crop development.

In reality, the subjective distinction between a dry spell and a genuine drought is a difficult one to make. Most American farmers do not call a dry spell a drought until the moisture shortage has seriously affected the established economy of the region. These observations apply equally well to similar situations in arid areas. It has been suggested that there should be at least two components to a definition of drought: a physical one and a social one. A mix of dry spell and meteorological drought occurred at various times and various places in the Sahel between 1968 and 1974, but their impacts were worsened by the pressure of populations, both human and livestock.

A major problem associated with the perception of drought is the constantly changing nature of those perceptions. Recent weather tends to influence perceptions more heavily than earlier weather and wet spells more heavily than dry ones. On this basis it has been suggested that the inhabitants of the Sahel have adopted practices during the abnormally wet years which end in disaster during the dry years. After a period of abnormally high rainfall in the Sahel which ended about 1965, the inhabitants considered a return to average conditions for rainfall to be a 'drought'.

What, then, does a return to average rainfall conditions in arid and semiarid regions mean? Of what importance is it when a return to 'normal' or near normal rainfall is perceived to be a return to drought-like conditions? Take the case of the Sahel, a climatic zone in sub-Saharan Africa which receives 200–600 mm of annual mean rainfall. It is bordered on the north by subdesert (100–200 mm) and to the south by the Sudan (600–900 mm). Rain falls during

August precipitation 1941–75



convective thunderstorms over a four-month period beginning in June. It is this highly variable rainfall on which the region's inhabitants depend for the cultivation of their crops and the maintenance of their herds.

August rainfall data are significant in that August is nearly always the wettest month and it marks the midpoint of the rainy season. We have looked at rainfall statistics for arid zones by taking the August rainfall records for two cities within the Sahel—Gao, Mali (16.3°N, 0.1°W) and Niamey, Niger (13.5°N, 2.1°E)—and have found that Sahelian rainfall shows a relatively high degree of variability. Many analyses, however, have not included a plot of the actual data but have relied on the use of descriptive statistical terms such as the mean or the running mean. Such terms fail to reveal magnitude and variability which are major factors in understanding the nature of rainfall in arid and semiarid lands.

Histograms derived from the data for Gao and Niamey show a 'positively skewed' frequency distribution, small amounts of rainfall occurring more frequently than relatively large amounts. This type of skewness is characteristic of the rainfall distribution not only in the Sahel but, more generally, in arid and semiarid regions. In particular, the degree of skewness is greater the drier the climate.

The mean, which is commonly taken to represent 'normal' rainfall, is extremely sensitive to skewness because it is inflated by the relatively few cases of large values: more than half of the observations fall below the mean. As a result, rainfall observations close to the mean value do not occur often in the Sahel, showing that, at least in some sense, the mean is too large and not at all indicative of how much rain commonly falls.

The median marks the amount of precipitation which is exceeded one half of the time, while the mode represents the amount of precipitation which is most likely to occur. Since neither of these measures is greatly affected by skewness, their interpretations remain clear. In particular, for a positively skewed distribution they are usually smaller in value than the mean.

For the Sahel, therefore, the mean, in reality, is above 'normal', while the median and the mode can still be interpreted, in the sense just discussed, as 'normal'. Hence although most analyses of Sahelian rainfall assume that the mean is an appropriate measure of central tendency, either the median or the mode may, depending on the circumstances, be preferable and no single number can adequately represent the distribution of Sahelian rainfall, given the large deviations from each of the measures of central tendency. 'Normal' rainfall, however, can be better characterised by the interquartile range than by any of the standard measures of central tendency.

Measuring the variability of Sahelian rainfall is further complicated by the possibility of trends or cycles or other evidence of climatic change in the data: there is strong disagreement among researchers over whether significant trends or cycles do exist. Although they might well exist, there may have been an inordinate degree of effort devoted to 'hidden periodicity' research and this has tended to obscure the most important aspect of Sahelian rainfall—its extreme variability. In fact, even if trends or cycles do exist, it is almost universally agreed that they would be of limited predictive value.

Aside from the absolute amounts of rainfall, as measured over varying lengths of time, other factors such as spatial distribution, timing, wind velocity and evapotranspiration could have an important impact on the social value to the inhabitants of the rain that does fall. If rainfall information, which represents only one climatic variable, is not viewed within its total environmental context, it can be extremely misleading. Where long-range development planning is concerned, such an oversight can be disastrous. Such has often been the case in fragile ecosystems like those in

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Camera Press

arid and semiarid regions.

Desertification—the creation of desert-like conditions where none had existed before—is the result of either the vagaries of weather and climate or the mismanagement of the land or, as in most cases, some combination of both. Such ecological deterioration in the Sahel has been linked in several ways to the increased sizes of livestock herds. During extremely favourable periods of rainfall, as was the fifteen-year period preceding 1968, the pastoralists moved into the marginal regions in the north with relatively large herds. However, with the onset of a series of dry years beginning at the end of the rainy season in 1967, the pastoral populations found themselves overtaxing very marginal range-lands with the result that the nomads viewed themselves as victims of a natural disaster.

Deterioration and ultimately desertification in the Sahel and in similar ecosystems could be combated if an ecologically realistic carrying capacity for the rangelands was determined. Although there appears to be widespread acceptance that such a determination would be significant, there has been little agreement to date on how to make operational the notion of carrying capacity (defined as the amount of grazing stock that the pasture can support without deterioration of either the pasture or the stock). Should the carrying capacity be geared to the best, the average, or the poorest years? Which combination of statistical measures would be most meaningful for the planning of long-term development of rangelands? On which variables should such an assessment be based—vegetation, rainfall, soil, ground and surface water, or managerial capabilities? Such inconclusiveness within the scientific community, while understandable, creates confusion for the decision-makers, who often decide either to take no action or who decide that all scientific suggestions are of equal weight and, therefore, indiscriminately choose any one of those suggested. Given the downward spiral of land deterioration it becomes essential that an ecologically acceptable carrying capacity be established and enforced.

The mistaken idea that drought in the Sahel is an unexpected event has often been used to excuse the fact that long-range planning has failed to take rainfall variability into account. People blame the climate for agricultural failures in semiarid regions making it a scapegoat for faulty population and agricultural policies. It will therefore be crucial that decision-makers know what statistical and quasi-statistical measures actually mean: no single number can adequately describe the climate regime of an arid or semiarid region. Decision-makers must supplement such terms as the 'mean' with more informative statistical measures to characterise adequately the variability of the climate. An understanding of this high degree of variability will serve to remove one of the major obstacles to resolving the perennial problems of the Sahel and of other arid or semiarid regions. □

Going on the defensive

Constraints on the UK chemical industry are affecting research in its laboratories. John Manos describes how

THE chemical industry has suffered some nasty shocks in recent years. The basic economics of oil-based petrochemicals production has been severely shaken by rises in the oil price, while in other sectors the industry has had to face the onslaught of environmentalists and regulators.

Because the industry is more research-based than most, it is not surprising that these pressures should have had their effect in directing work in its laboratories. But they are not the only factors which have contributed to a major shift in research aims. In general, a growing maturity in the private industry's end products together with the above constraints (and the economic pressures of a recession) have meant that research effort is increasingly described as 'defensive', allowing companies to maintain their existing positions in the face of controls and inflation, while consolidating their expertise in established product areas.

Research is increasingly devoted to 'replacement' ventures, in which old processes, extravagant on energy and raw materials, are replaced by new processes economical in both. By applying a total design or total systems concept, the industry is re-training itself to satisfy this new long-range research aim. Also the paucity of totally new products which have provided a satisfactory return over the decade, has meant that less and less effort is devoted to 'blue sky' research and the research function is having to gear itself to satisfy more of private industry's short-term aims. That means fewer new drugs and pesticides and no really new plastics.

This applies to both those polymer products which showed spectacular growth over the past twenty years and to the higher value fine chemicals, drugs and agrochemicals whose development is more readily associated with broadly-based laboratory research aims. In both these areas less than 10% of research effort is now spent on the search for new products, the increasingly competitive nature of the business now requiring that 90% be spent on improvement and widening the range of products and on reducing costs.

The bulk petrochemicals industry, which has allowed the rapid growth of polymer production to take place, has reached a different sort of maturity. The petrochemicals industry believes

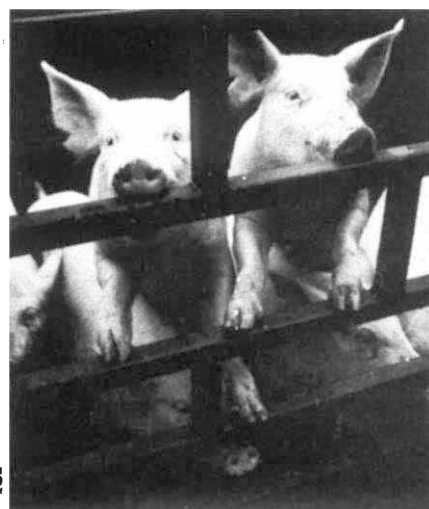
that Europe should have an energy policy that preserves for the chemical industry the chemical structures in 5% of total oil production, oil being too valuable to burn. If governments behave foolishly, the industry in Europe is considering which raw materials will replace (and to what extent) the cheap naphtha refinery by-product on which it has grown up. Research here is economic analysis of how best new feedstocks can fit in with established downstream production. While governments and state industries look at (or talk of) long term possibilities for the exploitation of new bulk raw materials, such as associated gas from North Sea oil wells or even coal, private companies are pre-occupied with justifying the operation of their existing facilities.

Biochemical engineering

If there is a pattern characterising research trends that is not symptomatic of the search for energy economy, pollution-free processes, or quickly exploitable product refinements, it is the opening up of entirely new areas of business based on the extension of chemistry into biology, and chemical engineering into biochemical engineering. It is surprising, for example, that each of the UK's big three chemical companies (and one of West Germany's) should have devoted such great research effort in seeking to move into the animal feedstuff industry. Yet this illustrates one of the chief areas of diversification: biochemical engineering.

In commercialising their bioproteins (or single cell protein) processes, BP, ICI and Germany's Hoechst are entering into a commodity business which is less well-known to them. In fact it is uncertainty about future price trends of the natural products which they aim partly to replace—soya bean and fish meal—which the third UK company, Shell, cites as the reason for its withdrawal from the development race. Nevertheless, 20% of ICI's 1977 capital investment sanctioning is devoted to a 50–75,000-ton-per-year plant to produce pig and poultry feed from hydrocarbon (methanol) feedstock. Methanol is also used in another animal feed development of ICI's at Billingham.

The bioproteins development programme has cost ICI £10 million over the past eight years with half spent in the last three. In its 1977 report, ICI



'Pruteen' piglets

describes this as a result of its 1960s initiative in applying process engineering know-how to microbiology. It is part of a bio-sciences research policy which the company has increasingly pursued in cooperation with universities. Other products of this line of research include processes for the continuous production of enzymes for the food industry (particularly isomerase for the production of high fructose corn syrup, for which ICI is now building a US manufacturing plant) and ICI's 'deep shaft' waste water treatment process which uses a mildly pressurised biochemical reaction to accelerate the conventional treatment process.

The forces which have encouraged the majors to venture into such unknown areas are the same as those which make research management in existing areas of business so difficult: the need for speedy exploitation of new developments countered by the increasingly long lead times to get new chemical products on the market. This latter point has been well documented for the agrochemicals industry where resources required to carry new insecticide through its seven- to nine-year path of screening, toxicology, field trials, registration and marketing are such as to restrict innovative development to only a small number of major companies.

Development effort

The development effort has swung over to what are generally less toxic products such as herbicides, or to the development of more efficient methods of applying existing compounds: ultra-fine sprays, slow release and encapsulated insecticides. Also newer synthetic pyrethroids under development are safer than the organophosphorous and carbamate products which are themselves safer than the organochlorines (which include DDT, aldrin and dieldrin).

Plant design and construction has been one area where companies have attempted to shorten development time. Shell Chemical, for example, has invested £19 million in a flexible agrochemicals production facility at Pernis, Rotterdam, which, although initially dedicated to specific products, incorporates in its design a service and equipment flexibility which will allow a switch to production of another product in as little as two months. But Shell still regards agrochemical development as a high-risk area from which resources will be diverted to more profitable business unless the regulatory pressures are eased.

The need to accelerate the worldwide exploitation of new developments is cited as one of the reasons why the overseas research budget of ICI has increased in recent years (from 25–30% of total in 1976). Thus, in the USA, ICI has extended its pharmaceuticals research effort so as to have the advantage of a scientific dialogue across the North Atlantic; and in India it is hoping to set up some central research facilities that will better use indigenous Indian raw materials within the Indian economy.

In general, however, rationalisation means centralisation, as illustrated by Hoechst and Shell's plans for their research facilities in Europe, although ICI still believes in a great deal of autonomy for its separate major business area. The greater part of Hoechst's investment in research facilities in recent years has been in its new multidisciplinary centre being built up in Frankfurt, which the company feels is necessary to cope with the rapidly changing demands of the market and of raw material supplies. Similarly last year Shell completed the transfer of all chemicals and polymers research facilities outside North America (except for agricultural pro-

ducts) to one site in Amsterdam.

As with agrochemicals, polymer research trends have been greatly influenced by the need to consolidate positions of strength and refine the properties of mature products. Hoechst notes that of the twelve plastics which represent 90% of Germany's consumption, not one has been developed within the last ten years. Polypropylene is the fastest growing new bulk plastic which threatens to compete strongly with the ethylene-based polyethylene, PVC and polystyrene (which account for 80% of German consumption) but this good rate of growth has come after twenty years of development! Other unique new materials such as carbon fibre, the wonder material of the sixties, have been overtaken by existing polymer technology which can satisfy rapidly changing customer demands more quickly and cheaply. The day of the accidentally-produced polymer having a market developed for it have passed.

The same accent on the selective development of established products was echoed by Shell last year in opening its polymer research centre in Amsterdam. It was suggested that the company, which had already withdrawn from two polymer areas (general-purpose rubber in the USA and high density polyethylene in the UK), might withdraw from other areas if its position was not strong. Certainly some polymer areas have shown a poor profitability recently. But as a number of products reach maturity over the next few years, Shell hopes that the concentration of product-oriented research capability at Amsterdam will allow a broadening of the range of applicability while process research is directed towards the development of improved, or second-generation processes for the production of the same polymers.

Non-productive research

Perhaps the most rapidly growing research area, however, is that involving non-productive work associated with toxicology and the control and detection of carcinogens. The complaints about over-regulation now heard from the agrochemical sector come more than a decade after the first problems associated with the use of agricultural chemicals caught the public eye. It was in the 1950s that concern over such products as the insecticide DNOC (dinitrocresol), which had caused the deaths of four farm workers, resulted in the setting up of the UK's Pesticide Safety Precaution Scheme. But increasingly stringent screening and animal testing techniques have always played a major part in product development in the agrochemical and the pharmaceutical industry. Exposure

limits and precautions for use of other chemicals not in contact with people or vegetation have generally been retrospective, based on experience of the chemicals in use.

This will not be so in the future. The 'ecology movement' and the need to avoid the difficult problem of dealing with a widely-used and manufactured product which is subsequently found to be dangerous and/or persistent in the environment will mean far tighter control on the introduction of all new chemical products. The US industry is already grappling with the massive requirements of the Toxic Substances Control Act and similar legislation is presently being formulated in Europe.

One of the biggest problems is the control of cancer-causing substances, or those which have given indication that they might be carcinogenic. The furore created by the US Food and Drug Administration's recent restrictions on the use of the sweetener saccharin and plastic soft drink bottles containing acrylonitrile indicates, according to the more pragmatic European view, the disadvantage of adopting a strict legalistic approach to the problem. But whatever the control strategy employed with known carcinogens, the problem remains simply how to screen new products for carcinogenicity to avoid the problem arising. With the several thousand new chemical products introduced every year being required to satisfy this sort of criterion, there is an urgent need for a simpler, quicker and cheaper screening procedure in preference to the conventional lengthy animal studies.

One of several possibly applicable *in vitro* tests is the Ames test, developed in the USA, which uses the mutation of bacteria strains as an indication of possible carcinogenic potential. For US companies such as Dow Chemical, currently spending 15% of its R&D budget on toxicology, such developments are of major importance in the light of new legislative requirements. But in Europe, too, *in vitro* testing is a major research area: ICI's central toxicology research laboratories have further developed the Ames test (in combination with another test involving mammalian cell transformation) to give the most reliable *in vitro* indication of carcinogenicity yet developed.

This exciting project is already justifying a budget of £250,000 a year which will rise to about £750,000 a year by 1978–9. Through such techniques, the company also sees a possible resolution of the problem of establishing no-effect levels for exposure to carcinogens, possibly within five years, □



Protein pilot plant

USA

Space in a corner

A tough fight is in prospect over two important space science projects for which funding is threatened. Colin Norman reports from Washington.

THE budget for the National Aeronautics and Space Administration (NASA) has run into unexpectedly heavy weather in its passage through Congress, and two major scientific projects could be in trouble. They are the Large Space Telescope, an orbiting optical telescope which has long been regarded by space scientists as NASA's top priority space science project, and the Jupiter Orbiter Probe, a proposal to launch twin spacecraft early in 1982 to orbit Jupiter and send probes into the planet's atmosphere.

A key subcommittee in the House of Representatives has already voted to block funds for the Jupiter project, and a sharp attack on the space telescope is expected in the Senate, with Senator William Proxmire leading the charge. Proxmire occupies a pivotal position since he is chairman of the subcommittee which is handling NASA's budget in the Senate; he is also armed with a new analysis suggesting that the total cost of building and operating the space telescope could run as high as \$1,400 million.

Though it is possible that both projects could be rescued by an intense lobbying campaign, already being mounted by NASA and its clients in the scientific community, loss of either of them would be a serious blow to the space science programme.

Both projects have been on the drawing board for some time, and NASA is now seeking Congressional approval to move them into the development stage. It has requested \$36 million for the space telescope and \$20.7 million for the Jupiter Orbiter Probe in fiscal year 1978. Those sums represent the first instalments toward the total development costs of \$435 million for the telescope and \$285 million for the Jupiter project, according to NASA's latest estimates.

Opposition from Senator Proxmire to the space telescope has long been anticipated, but the moves against the Jupiter project seem to have caught NASA officials by surprise. The first sign of trouble came late last month, when a House appropriations subcommittee deleted funds for the project from NASA's budget request. The subcommittee is said to have been unwilling to allow NASA to begin two

major projects in one year, and since subcommittee members had been under heavy pressure from space scientists to approve the telescope, they opted for that project and voted to kill off the Jupiter mission.

The subcommittee's recommendations will be reviewed by a meeting of the full House Appropriations Committee next week, but it is considered unlikely that the funds for the Jupiter project will be put back at that stage. Similarly, it would be unusual for the full House to overturn the committee's recommendation by restoring the funds. NASA is therefore concentrating its lobbying efforts on the Senate in the hope that if the Senate approves the project, House members can be persuaded to follow suit when differences between the House and Senate versions of the NASA budget bill are eventually settled in a House-Senate conference committee. The crucial vote will come in mid-June, when Proxmire's subcommittee is due to vote on NASA's budget bill, and the outcome is difficult to predict for a variety of reasons.

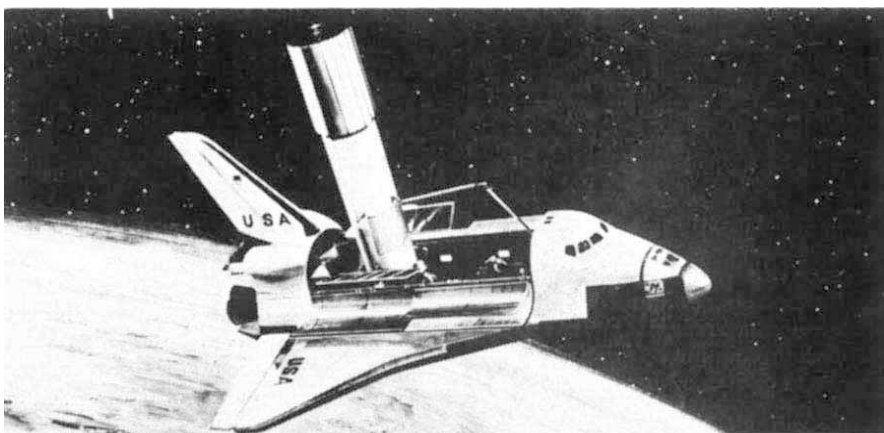
The chief focus of attack in the Senate is likely to be the space telescope, and Proxmire has already fired his opening shot. The Large Space Telescope (LST) has been under serious consideration by NASA since the early 1970s, and it has been consistently and enthusiastically endorsed by astronomers. Due to be launched by the space shuttle in 1983, the LST would have a major advantage over ground-based telescopes: the light it receives would not have to penetrate the semi-opaque curtain of the Earth's atmosphere, which filters out all but a narrow band of colours and blurs distant objects. As a result, the LST would be able to 'see' objects about 10 times smaller than those that can now be picked up

by the most powerful ground-based instruments, and it would be able to search for objects emitting light from the far infrared to the far ultraviolet, a range one thousand times larger than Earth-bound instruments.

Though the telescope has always been popular among scientists, it has not received unqualified support from Congress. A couple of years ago, before NASA had even asked Congressional approval for the LST, the House Committee on Science and Technology directed the agency to seek international participation in the project, to help defray some of the costs. Consequently, NASA has established a European connection. Last October, the Science Planning Council of the European Space Agency (ESA) approved a contribution of some \$80 million to the project, and according to NASA officials, a formal agreement will soon be signed under which ESA will supply a faint object camera, a solar array, a number of scientists for the project team, and assistance in maintaining and overhauling the telescope.

The LST has already been considerably delayed. NASA was originally hoping to begin building the instrument this fiscal year for launch in 1982, but the project collided with President Ford's attempts to hold down government spending. The Office of Management and Budget deleted funds for the LST from NASA's fiscal year 1977 budget before the budget was sent to Congress. According to NASA officials that delay has already hurt the project and a further delay would probably kill it. It would be difficult to keep the design teams together for much longer, they argue, and European support would probably melt away if Congressional approval isn't assured.

Proxmire opened his assault on the LST last week by releasing a report prepared by the General Accounting Office (GAO) which suggested that the



Space shuttle placement of LST

total cost of building the telescope and operating it for its projected 15-year lifetime could amount to \$1,400 million. "The country faces an \$80 billion deficit, and it is time the Congress said 'no'. The space telescope is a good place to start", said Proxmire.

The GAO essentially took NASA's estimate for \$435 million to develop the LST, and added \$96 million for inflation, \$65 million for tracking and data acquisition, \$470 million for general operations costs, \$81 million for civil service personnel, and \$211 million for shuttle launches to put the instrument into orbit and to refurbish and maintain it.

The GAO figures have sent NASA officials into orbit. In a letter printed in the GAO report, Noel Hinners, Associate NASA Administrator for Space Science, charged that "presenting this eclectic array of cost data as a life-cycle cost estimate for the space telescope is misleading". In an interview with *Nature* last week, Hinners said that NASA is still estimating that the development cost for the LST will be about \$435 million, and that the operations costs will be about \$10-\$15 million a year. The operating estimates do not include shuttle launch and refurbishment, he acknowledged, because it is not known how frequently the telescope will be visited for maintenance.

Hinners argued that the operating cost for the LST will not be much different from the \$10 million it costs to operate a major ground-based facility such as Kitt Peak. "I don't see anybody saying that if Kitt Peak lasts for 100 years it will cost \$1 billion", Hinners wryly observed.

Nevertheless, whatever the accuracy of the GAO figures, the report highlights the fact that the cost of the LST will not end with the launch, and the figure of \$1,400 million is sure to be a powerful weapon in Proxmire's hands. Few people are willing to predict the outcome in the Senate for, although Proxmire has failed to sway his committee against NASA projects in the past, there has been a significant change of membership on his committee this year. Two staunch supporters of the space programme, Lawton Chiles of Florida and Bennett Johnston of Louisiana, have been replaced by James Sasser of Tennessee and Patrick Leahy of Vermont, both of whom are fiscal conservatives. Even if he loses in committee, however, Proxmire is expected to take his fight to the Senate floor.

According to committee sources Proxmire is concentrating on the LST at this stage, and is probably prepared to approve funds for the Jupiter Orbiter Probe (JOP). But the sources

suggest that if Proxmire loses on the LST, he may then turn on the Jupiter project. NASA officials would regard loss of that project as a body blow for the planetary science programme in general and to the Jet Propulsion Laboratory in particular. If both the House and Senate delete funds for the project, there would be no chance to reinstate them in conference committees.

At present, there is only one approved planetary mission under way. That is the so-called Voyager project which involves launching spacecraft in August this year to flit past Jupiter and travel on to Saturn. One of the spacecraft may also be re-targeted to swing past Uranus eight years after launch. Thus, if the JOP is not approved, there would be a major hiatus in the planetary programme, with no projects under development at all. According to NASA officials, up to 300 people would be laid off at the JPL, which is now the focus of NASA's planetary work. It should be noted that if the 1982 launch date is missed, there would not be another good opportunity until 1987, because Jupiter will be too far away.

Again, NASA officials argue that if the JOP is killed, planetary science teams would be broken up, and the continuity between programmes would be lost. The technology used on the Pioneer Venus probes would be adapted to the JOP mission, and the JOP spacecraft technology would in turn be used on the planned out-of-ecliptic mission. If there is a major disruption in the planetary programme, "we would lose that inheritance from one mission to the next", Hinners says.

NASA officials are taking those arguments to Senators now in the hope of building support for the efforts. Though the prospects are difficult to assess, it is expected that the LST will scrape through in the Senate, and that the Senate will also approve some funds for the JOP. If those decisions are upheld in the conference committee, NASA would be able to start work on JOP next year, and avoid major layoffs.

Finally, it should be noted that the reason why these projects are deemed especially crucial this year is because some space science projects have been squeezed out of NASA's budget during the past four years to accommodate funding for the space shuttle. Space scientists have been assuming, however, that when funding for the shuttle begins to run down, more money would be made available for space science. Next year is the first year of reduced shuttle funding, but science projects are still at the front of the firing line. □

DNA bills drafted

UNDER intense lobbying from a number of scientists, committee staff members in the House and Senate have put together draft versions of legislation to control recombinant DNA research in the United States. Though there are still plenty of opportunities for the drafts to be altered by the committees themselves, major differences are shaping up in the approaches embodied in the House and Senate versions. The draft Senate bill, in particular, is causing consternation among many scientists.

The House bill has already been approved in principle by the health subcommittee of the House Commerce committee, and members of the subcommittee are expected to meet next week to approve the final wording. It will then go to the full committee for approval before being sent to the floor of the House. The Senate version has not progressed that far. It has been drafted by staff members of the health subcommittee, though it is said to reflect the views of the subcommittee chairman Edward Kennedy, and it will not be considered by the committee itself until next week at the earliest.

The House draft would require the Secretary of Health, Education and Welfare (HEW) to draw up new recombinant DNA regulations, with the advice of a top-level advisory committee, and to licence facilities where such experiments are to be conducted. The bill would leave much of the implication of the regulations, including the granting of most licences, to local biohazards committees, however. Consisting mostly of scientists, the committees would include lay members and representatives of local communities. The Senate version, on the other hand, would establish an eleven-member commission in HEW to draft regulations and issue licences for facilities and for individual experiments. Both versions imply that, until new regulations are adopted, guidelines issued last year by the National Institutes of Health would be given the force of law.

The Senate approach has come under heavy fire by scientists, most conspicuously in a resolution approved late last month by the National Academy of Sciences, for fear that it would lead to bureaucratic delays and subject the research to inordinate red tape.

One of the most controversial issues in the debate on federal legislation has been whether state and local governments should be allowed to establish local controls which are more strict than the federal regulations. The draft House bill would allow them to do so only if they can prove to the Secretary of HEW that there are compelling local reasons for stricter controls, or that the federal regulations are not strict enough to protect public health, requirements which come close to making the federal controls paramount. In the Senate, Kennedy is said to favour allowing state governments to set stricter controls, though some other members of his subcommittee favour the House approach and agreement has yet to be reached.

Colin Norman

BRITAIN

Waiting for summer

Chris Sherwell reports on latest developments in Britain's nuclear power debate, now picking up again

THE issue continues to insinuate itself; only its locus changes. The excitement over nuclear power in recent weeks—since President Carter announced his policy—has been generated in various international forums, notably in London at the meeting of the so-called nuclear suppliers group and at the Downing Street economic summit, and in Salzburg, where two conferences were held in quick succession.

Now the issue is coming home. The second Salzburg conference had not even ended late last week before British experts were called away to attend one of the two meetings which took place in London at the weekend on domestic nuclear policy. The one bringing the experts back was called by Mr Anthony Wedgwood Benn, the UK Secretary of State for Energy, and was held in secret. Ironically, the other meeting, held in public and involving hundreds of largely lay people, attracted far less publicity. It was organised by Friends of the Earth (FoE).

Called a conference for a non-nuclear world, the FoE meeting produced a mostly young audience whose views on nuclear power were much as expected: negative. Their enthusiasm was fanned by remarks from two MPs, Robin Cook and Nigel Forman. Cook urged local groups to latch on to nuclear-related local issues and to exploit them relentlessly; Forman's advice was simple—citing a recent opinion poll, he said "secure yourself the help of a friendly scientist".

Neither man was invited to Benn's meeting, though the Commons was represented, through the chairman of the Select Committee on Science and Technology, Arthur Palmer. Indeed, many of the 30 or more people meeting in the seclusion of the Civil Service College at Sunningdale were favourably disposed to nuclear power. They included Sir John Hill of the UKAEA, Con Allday of BNFL, Ned Franklin of the National Nuclear Corporation (NNC), and Frank Tombs of the Electricity Council. Others present included Sir George Porter, Sir Alan Cottrell, Lord Todd, Sir Herman Bondi, Sir Kenneth Berrill, Lord Balogh, Dr Walter Marshall and Sir Brian Flowers—altogether a high-powered line-up.

That not all were so disposed—Walter Patterson of FoE was there to

put the alternative view—at least said something about Mr Benn's desire to hear all sides of opinion. And opinions were what he was there to hear: it was not a meeting to take decisions; rather, it was a meeting that anticipated them.

After months if not years of the public debate he has himself spurred on, Mr Benn is now coming under increasing pressure to call a halt to it all and to start, in the words of one participant at Sunningdale, doing what he was elected to do—take decisions. With the inquiry on an oxide fuel reprocessing plant at Windscale for BNFL due to begin on 14 June, that controversial issue is temporarily out of his hands. But two others await action.

One is on the choice of nuclear reactor for Britain, an issue which goes back to at least 1974, when everyone thought it was finally agreed that the Steam Generating Heavy Water Reactor (SGHWR) would be the country's next generation reactor. The issue was reopened last year, and decision-time has again arrived. The choice is between the SGHWR, the Advanced Gas-Cooled Reactor (AGR), a British product now apparently performing well after teething troubles, and the Pressurised Water Reactors (PWR) which now dominate the world market.

Press reports over the past couple of months have revealed a tilt in favour of the AGR. Mr Benn is himself awaiting a detailed report from the National Nuclear Corporation on the

three types of reactor, and has advised its compilers of the sort of questions he wants answered. He is also awaiting a report from the National Nuclear Inspectorate on the safety of PWRs. Both reports are due out soon, probably next month.

At the weekend meeting Sir Brian Flowers, the former chairman of the Royal Commission whose report last year on nuclear power became the layman's *locus classicus*, urged that a choice be made. That report, the government's response to which is also due to be published shortly, escalated the debate on the fast breeder, which is the subject of Mr Benn's other decision. Having originally promised a decision last autumn and then stated earlier this year that a decision yet is not necessary, Mr Benn is now talking about the coming autumn as a deadline. The decision is on whether to go ahead with a demonstration commercial fast breeder (CFR1) at Dounreay, and the industry, notably in the form of Sir John Hill, is saying that there is no time to waste.

All the suggestions of urgency, and there are now many, do not derive from an immediate need for energy. The worry is that the growing surplus Britain is now acquiring will be lost by the 1990s, and that long lead times make action now essential if nuclear power is to play its allotted role alongside coal and conservation as one of the principal originators of Britain's supplies in the 1990s. That nuclear power is destined to do that was apparently agreed at Sunningdale; that it should entail a full nuclear cycle and

After Moscow, the world

EVERYONE knows the world market for energy has been transformed in this decade. Not everyone appreciates that the world market for energy (and every other commodity) could be transformed completely and even more radically within the next couple of decades if China and the USSR, separately or together, produce the sort of economic growth that countries like Japan or Germany have experienced.

One man who does claim to appreciate the problem is Britain's Secretary of State for Energy, Mr Anthony Wedgwood Benn. Last week he spent two full days in Moscow and came away enthralled. His purpose was to paint in the detail of the 'eastern perspective' on the global energy canvas that he likes to speak about. To get the other perspectives—west, north and south—he has already visited Washington, Norway and Saudi Arabia. Having assimilated all that, his next step will come at the EEC Council of Energy Ministers in the middle of June, when he reports back.

His tentative conclusion is that there is a need for a world energy policy.

What this means precisely is not clear: to many, a world policy implies a world government to implement it; to Mr Benn it means at the very least having information on world supply and demand for energy. He cites almost scornfully a book out this week on global energy prospects to the end of the century which takes no account of countries like China or the Soviet Union.

So what did he learn in Moscow? That the USSR is the world's biggest energy producer. That the Russians hope to use still higher voltage electricity transmission. That they are interested in Britain's offshore oil technology. That they do not dump medium-level nuclear waste into the sea. And that they are very keen to hear assessments of US nuclear policy.

Mr Benn also received one "small rebuke". Each year, he was told, 100 US scientists visited the USSR, and 100 from the USSR visited the States. For France the figure was 70. For Britain over a three-year period, however, the figure was only 50.

Chris Sherwell

a system of fast breeders was a matter of greater dispute.

From all accounts the participants agreed that if reprocessing was to be done at all, it could be done most reliably at Windscale. There was also fairly wide agreement that technically speaking enough could be done to combat the threat of proliferation, but that the problem of public acceptability—the all-important political dimension

—would remain. Had any of the participants retained doubts on that score, the other weekend meeting might have helped to dispel them.

Now, with few new arguments to present or to hear, everyone with any influence over the shape of Britain's nuclear policy is hoping for just one thing: that with decisions ultimately a matter only of a signature, Mr Benn will have a restful summer. □

SWITZERLAND

Guidelines emerge

Rosmarie Waldner reports from Zurich on Swiss moves to produce guidelines for genetic manipulation experiments

A Swiss Academy of Medical Sciences Committee founded in 1975, the Commission for Experimental Genetics, recently took the first step towards regulating genetic manipulation in Switzerland when it sent a circular to all researchers in the field of recombinant DNA recommending observance of the guidelines laid down by the US National Institutes of Health (NIH), Switzerland. It sent a circular to the guidelines issued for NIH researchers in June last year. The Swiss guidelines, however, are not legally binding, although they are considered to be obligatory for the researcher. The committee also wants research projects to be registered, in line with the recommendations of the Strasbourg-based European Science Foundation (ESF).

The commission, under the presidency of Professor Werner Arber of the Biozentrum of the University of Basle, is of the opinion that "things as they stand at present do not justify issuing an official order. The caution expected of researchers in this field, with its legal basis in the Epidemic Law, should ensure general observance of the recommended guidelines". The Swiss Epidemic Law regulates measures in connection with infectious diseases and is one of the few laws in the field of public health valid in all parts of Switzerland; public health, not being a federal matter, is under the jurisdiction of Switzerland's 25 cantons.

At their annual conference held in early April the Swiss Society for Cellular and Molecular Biology discussed the guidelines and voted overwhelmingly in favour in them. However, some speakers demanded that the guidelines be made legally binding and that the public should be brought into the discussion. Altogether six laboratories at the universities of Basle, Geneva and Zurich and at the im-

munology institute of the pharmaceutical company Hoffman-La Roche in Basle are working with recombinant DNA.

The commission is available for advice or will arrange for expert assistance to be provided by the European Molecular Biology Organisation (EMBO) in Heidelberg if local researchers have any doubts about the correct classification of a research project within the various criteria of the NIH guidelines. It is also available for general consultation and for the interpretation of the NIH guidelines. As Professor Arber stresses, the commission does not consider the NIH guidelines adopted in Switzerland as unalterable; it wants to keep a constant watch and make changes (even if these deviated from the NIH guidelines) if this was considered necessary.

When registering, researchers responsible for a project must announce their intentions and state the precautionary measures taken. "The committee considers the observance of these measures, registration and the provision of correct information to colleagues to be part of the duty of each and every researcher", the circular states. Research heads and heads of department at universities and other public and private institutions have been requested to support the commission's efforts.

The Swiss National Fund for the Promotion of Scientific Research particularly wants to promote the research in Switzerland. When it comes to the allocation of research funds, however, it insists on observance of the guidelines. The fund has mentioned the possibility of becoming involved in the establishment of a special safety (P4) laboratory. This project is expected to cost SF1.5 million.

The Swiss press has welcomed the guidelines, but there have been complaints that decisions concerning research with such far-reaching consequences should have been made in camera—the commission has no lay representatives, nor even experts from

W. GERMANY

Breeder hold-up

UNDER increasing pressure from environmental protectionists, the West German government has called a temporary halt to research and development on the fast breeder reactor. Last week, Dr Hans Matthöfer, the West German Minister for Science and Technology, announced that no more money was to be spent on R&D for the fast breeder until questions raised by the Social Democrats on the safety of fast breeder technology and the proliferation of nuclear weapons through the spread of plutonium had been considered by the Bundestag.

This means a bar on spending whatever has not yet been committed of the DM122 million set aside for fast breeder R&D under the government's medium term energy plan. Work already contracted, however, such as the prototype liquid metal fast breeder at Kalkar, will go ahead. It will be financed from that portion of the DM227 million fast breeder programme not devoted to research. Other projects unlikely to be axed are West Germany's collaboration in the construction of the French Superphénix and the fabrication plant for fast-breeder fuel elements which is already in operation.

Prior to the latest announcement discussion about the further development of breeder reactors in West Germany was becoming livelier. In the Social Democratic party above all there was increasing resistance to further financing of R&D work. The Ministry for Research and Technology had explained in detail to the Bundestag the pros and cons of the evolution of the fast breeder—it seems unsuccessfully.

Germany began development of the breeder in 1960—researchers got their early experience through participation in the construction of the American experimental reactor SEFOR. The 300 MW breeder at Kalkar is a German-Belgian-Dutch joint project due to be completed in 1982. Within the framework of the German-French agreement on Superphénix, Germany contributes 16% of the cost via electricity supply undertakings.

When the West German government presented its energy research programme at the end of April, Dr Matthöfer claimed that because of public resistance, his ministry had felt obliged not to balance the programme as much in favour of nuclear power as it had originally intended. It had been necessary to cut the target of 45,000 MW of nuclear power by 1985 to 30,000 MW. Nevertheless, the energy research programme puts greatest emphasis on nuclear power: out of the DM6,200 million to be spent on energy up to 1980, DM4,500 million was to go on nuclear energy. □

fields other than biology and medicine. The hope was expressed that parliamentarians would soon be roused: questions were put in parliament as early as two years ago; since then there has been silence on the subject.

IN BRIEF

US nuclear snag

President Carter's plans to defer nuclear fuel reprocessing and to turn down the breeder reactor programme have hit a snag in the US House of Representatives and they could face a stiff challenge in the Senate. Last week, the House Committee on Science and Technology voted 38 to 0 to keep funds for a demonstration breeder reactor in the budget for the Energy Research and Development Administration (ERDA), but with the understanding that the committee will investigate the matter further and vote on the funds again before sending the ERDA budget bill to the House floor.

Committee sources note that the vote was a procedural measure which doesn't necessarily indicate the committee's final view, though they suggest that the prevailing opinion among committee members seems at present to favour rejecting Carter's request to cancel the demonstration project. In the Senate, Senator Frank Church, who heads a key energy subcommittee, said in a speech at MIT earlier this month that Carter's plutonium strategy will not work because other countries are unlikely to follow suit. In a later interview with the *Washington Post*, Church said that the United States should continue with the breeder project and with reprocessing to avoid

"nuclear isolationism". The opposition to Carter's plans virtually guarantees that there will be a major debate on plutonium policy on the floor of the House and the Senate this summer.

Pipeline report

Controversial recommendations from a Canadian judge regarding proposed pipelines stretching between the Arctic and Southern Canada and the USA will not be acted upon at least until a second report is published, probably in the summer. The judge, Thomas Berger, reported on the ecological and social implications of the proposal last week, and recommended a morator-

THE heavy veils of secrecy that the French are drawing over the details of their method of enriching uranium to reactor- but not to weapons-grade make it difficult to evaluate their claims. But if their recently revealed chemical enrichment technique stands up to full scale testing and is as tamper-proof as they suggest, cheap enrichment plant should be available in the 1990s to many countries which might not have been considered responsible enough to own it.

The French announcement, at the recent IAEA nuclear fuel cycle conference in Salzburg, was neatly timed to coincide with the economic summit in London. With an undoubted shortage of enrichment plants the new process seems to offer the possibility of meeting the need without increasing the risk of proliferation. It is not clear, however, whether such enrichment facilities would allow the use of the fast breeder reactor to be postponed; that depends on whether, in the absence of the new technique, the use of uranium in thermal reactors would be limited by a shortage of enrichment facilities or by the scarcity of uranium itself—which in turn will be determined by the economics of extraction.

Enrichment used to receive a great deal of attention as a process encouraging nuclear proliferation. Concern has since focused on reprocessing the spent fuel. Natural uranium, containing 99.3% ^{238}U and 0.7% fissile ^{235}U , must be enriched to 3–4% ^{235}U for use in American LWRs and 2% for the British AGRs. The Canadian CANDU and the British Magnox reactors use natural uranium, as oxide and metal respectively. The sensitive aspect of enrichment is that an enrichment plant may be arranged so that enrichment to over 90% instead of 2–4% is

achieved, enough to make a uranium bomb.

Reprocessing offers a similar opportunity, but here to make a plutonium bomb. The civil purpose of reprocessing spent reactor fuel—that is, separating plutonium from uranium and radioactive wastes—is to use the

Power sans bombs

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BACKGROUNDER

plutonium to fuel more reactors and especially the fast breeder. The French development could make it more difficult to get hold of uranium bombs, but would not affect the ease of access to plutonium bombs.

The main enrichment process in current use is gaseous diffusion, uranium hexafluoride being diffused through a porous membrane. The lighter ^{235}U atoms diffuse slightly more readily than the ^{238}U and enrichment is achieved by a large number of stages in cascade. Compression and cooling at each stage mean that energy consumption is very high (6% of the energy generated by PWR fuel). In the newer ultracentrifugation process, favoured by the British, Germans and Dutch, the

diffuser is replaced by a centrifuge. The capital costs of centrifugation are very high but the electricity consumption is only 10% that of diffusion.

The chemical exchange process announced by the French is said to have the advantages of low energy consumption, rather simple technology and suitability for small-scale plants. Separation of the two uranium isotopes based on the different rates with which molecules containing them react has been explored in the past, but had been largely discounted by most workers because the very small difference in chemical reaction rate only allows extremely slow separation.

The French breakthrough apparently lies in finding two uranium compounds which undergo reactions whose rates are more markedly affected by the uranium isotope. What the molecules are has not been revealed. The enrichment process is still rather slow but this is the main safeguard against bomb manufacture; while the period of two years needed to produce 3% enriched uranium by the chemical method seems industrially feasible, it would take fully 30 years to produce bomb-grade plutonium. By contrast, bomb-grade material can be prepared in two years by gaseous diffusion and one day by ultracentrifugation.

The French say two other safeguards are built into their process. One is that the process cannot be arranged in cascades (which can, at least in theory, be manipulated to produce a small quantity of 90% enriched fuel instead of a larger quantity of 3% enriched fuel). Furthermore, if too much enrichment is attempted, the uranium risks going critical. This seems to be because a liquid phase is involved, and in a liquid there is the risk of local concentrations of very high enrichment.

ium, to the chagrin of industrial interests. A second report on the economic need for the pipeline is awaited from the Canadian National Energy Board, which regulates the oil and gas industries and is still holding hearings.

USSR action on seminar

Professor Mark Azbel has been ordered by the Soviet authorities to cease holding the 'Sunday Seminars' for refusenik scientists. The seminars have been held regularly for five years, and only a month ago Professor Azbel was given a once-off permission to hold a special anniversary session—the first time that the authorities openly acknowledged the existence of the seminars.

Within the past three weeks, Pro-

fessor Azbel has been summoned three times to the Lubyanka and Lefortovo prisons for interrogation. He was obliged to sign an undertaking not to reveal the contents of the investigation. It is known, however, that it related to his association with Anatolii Shcharanskii, the unofficial 'press-agent' of the seminar group at present held for investigation for alleged contact with the CIA.

Last week Dr Viktor Brailovskii and Dr Veniamin Fain, who are among the senior members of the group, were also called in for interrogation.

The most recent message received from Professor Azbel was that the authorities are striking at the 'Professorate'. Previous action against refusenik scientists has been aimed at the lower echelons.

NPL's new director

Dr Paul Dean is to take over as director of the National Physical Laboratory (NPL) on 1 October. He will succeed Sir Iuean Maddock, who has been acting director since giving up his appointment as chief scientist at the Department of Industry at the end of last March. Dr John Dunworth, the previous permanent director, retired last June; he joined the NPL as deputy director in 1962 and became responsible for the laboratory in 1964. Dean joined the laboratory in 1957, and will be moving from the Department of Industry's headquarters, where he was head of the Space and Air Research division. He was also Head of the Research and Development Contractors division.

THEY came in busloads from as far away as Minnesota. They wore pro-laetrile buttons. They were prepared to pack the hearing room, to applaud wildly at the inflammatory oratory of their leaders, and to boo, hiss and interrupt when members of the 'medical establishment' warned them of the uselessness of laetrile in cancer therapy.

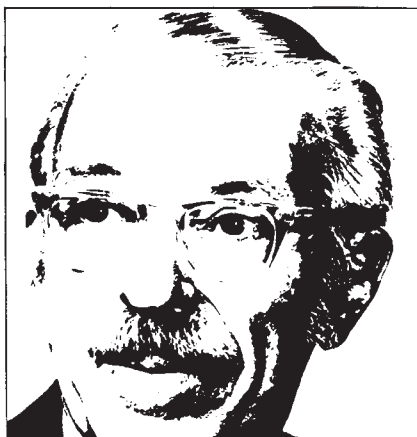
The occasion was the Food and Drug Administration (FDA) Administrative Rulemaking Hearing on Laetrile, held in Kansas City, Missouri at the beginning of May. To 'bend over backwards', the FDA had talks by the laetrile crusaders, following opening remarks by the presiding officer. My experience with hearings led me to know that the television cameras and reporters would be present at the morning session, and they would depart at noon, leaving the opponents of laetrile without media coverage, just as the opponents of banning DDT were treated in 1969.

Efforts at appeasement always fail. At 8 am on the first day the laetrile pushers called a press conference that was largely devoted to rhetoric against the FDA and allegations that the hearings were a "kangaroo court". I asked if the laetrile supporters had been given the opportunity to speak at the hearings. The answer was, necessarily, "yes", but a woman hastened to my side and hissed in my ear that if I interrupted their press conference with any more questions, she would call the hotel manager and have me removed forcibly from the room. I managed to avoid this fate when I asked my next question, by alleging that I was a reporter for *Nature*. I requested any scientific literature reference, even one, to substantiate the claim that laetrile is

vitamin B17. I was told "wait until tomorrow".

During the hearings, professors of medicine were jeered at and heckled. Advocates of laetrile, shouting and declaiming in the manner of a

Quackery triumph



THOMAS H. JUKES

religious revival meeting, were wildly applauded as they testified to their "cures". The audience was ecstatic, as well it might be. Bills to legalise laetrile without even defining what laetrile actually is, have just been passed by the legislatures of Alaska, Florida, Indiana and Nevada. The Indiana Bill had successfully over-ridden the Governor's veto. The Nevada bill includes a 10% cut on laetrile sale for the State Treasury.

The publication of the laetrile pushers, *The Choice*, was bursting with exultant information. It contained advertisements for "Reverend Clifford Oden's stirring call to faith in God and vitamin B17", for a new

water distiller that would remove fluoride from drinking water, and for a book on "chelation therapy in the killer diseases". The publication informed its readers that "efforts to decriminalise laetrile use in Texas surged into the forefront where 150 members of the lower house shouted their approval of the freedom of choice bill and stood up and cheered after the unanimous vote was delivered". One of the photographs showed two 'Wisconsin committee leaders' carrying "23,000 names on signatures" in support of pro-laetrile bills introduced into the Wisconsin legislature; another was of Gloria Swanson telling her health philosophy and her personal victory over a tumour.

The pro-laetrile campaign is administering a crushing defeat to science and rationality. Undoubtedly it is well-financed. A Californian 'laetrile physician' was indicted on charges including one of depositing \$2,500,000 in California banks, May 1973 to August 1975. His licence was revoked for "gross negligence and incompetence", 29 November, 1976.

The technique for arousing support is an appeal to revolt against authority, bureaucracy and the 'medical establishment'. Political action is effected by writing letters to legislators. This procedure was overwhelmingly successful with the Proxmire Bill in the US Senate 1975, specifically directed against the authority of the FDA.

Things fall apart; the centre cannot hold;
Mere anarchy is loosed upon the world,
The blood-dimmed tide is loosed, and
everywhere

The ceremony of innocence is doomed;
The best lack all conviction, while the
worst

Are full of passionate intensity.

W. B. Yeats

correspondence

Technology and politics

SIR,—After making several criticisms of *The Technology of Political Control*, your editorial (14 April, page 577) ends with a conclusion which displays an apparent ignorance of the structural basis of control. Although I accept the admirable sentiment that, "the real need is for scientists who are involved in military and police research to be able to speak up when they see the balance shifting in an unsavoury direction," the realities of the situation in practice make this suggestion naive. Given the essence and ramifications of such work, it is likely to be covered under the Official Secrets Act and thus government researchers in these areas are forbidden from making their work public.

The writers of the *New Technology of Repression* are among the few scientists who can present unrestricted information on this topic in an informed way. Yet, as the recent search of my house and university room by the Special Branch indicate; no-one who researches in this controversial area can be assured of freedom from interference. It must not be forgotten that scientists are also numbered amongst the victims of the technologies of political control.

Yours faithfully,

STEVE WRIGHT

Department of Politics,
University of Lancaster, UK

'The Selfish Gene'

SIR,—It is no great surprise that W. D. Hamilton has written such an intemperate letter about my review of *The Selfish Gene* (12 May, page 102). Despite his considerable concrete contribution to the theory of kin selection, Dr Hamilton has himself given way to the lure of unscientific speculation on the biological basis of human history, and is responsible for his fair share of vulgar Darwinising. I will comment on three points in his letter that have appeared frequently in replies to criticisms such as mine. First, despite a string of pejorative comments on my objectivity and the quality of my analysis not a single instance is offered of my mistaken analysis. The reader will be as suspicious as I am that "It would be easy to defend the book and reply to the review point by point" when not a word of defence is offered, but

only 350 words of angry invective.

Second, with great modesty, Dr Hamilton compares himself and his fellow sociobiologists to Darwin, and Dr Dawkins to Huxley. Other biological determinists have recently, in their apologies, compared themselves to Galileo! Everyone with a pet theory thinks himself a Darwin or Galileo, the moment his errors are criticised. But my criticism of Dawkins comes not from an outraged church establishment, but from the solid accumulation of the science of population genetics. The real problem in scientific progress is to distinguish those with a revolutionary new insight into nature from crackpots with delusions of grandeur. Self-advertisement is poor evidence.

Finally, Dr Hamilton confesses that he feels little concern about the basic epistemological questions that are at issue. Rather he feels a great warmth about the "spirit" of Dr Dawkins' book and the "science" it describes. But it is precisely the substitution of "spirit" for the elements of logical thought that makes *The Selfish Gene* such a worthless piece of vulgarisation. What is revealed by Dr Hamilton's letter is that Dawkins' errors are not simply those of an overenthusiastic but naive populariser. They are an accurate reflection, it seems, of the quality of thought of the sociobiologists themselves. We will surely not understand the nature of the world by an excess of spirit and a deficiency of hard thought. Dr Hamilton, Bishop Wilberforce would have been proud of you.

Yours faithfully,

R. C. LEWONTIN

Museum of Comparative Zoology,
Harvard University,
USA

Scotland get its share

SIR,—It is difficult to know whether the letter by D. N. M. Hamilton (28 April, page 770) is meant as a serious contribution or as a joke. If he seriously thinks that those who advise the Medical Research Council state that the Scots believe that the people in London are more deserving of money for research than are the research workers in Scotland, I should have thought he would have drawn this to the attention of the MRC directly and suggested most strongly that they change their advisers.

My department has been generously

supported and assisted by the MRC on the basis of projects submitted to them, and in my many conversations with the administrators in the MRC, I have been left in no doubt that there is considerable regard for the immense amount of scientific activity that is conducted in the scientific centres in Scotland. At no time during any conversation have I been left with the impression that institutions outside London—and particularly those North of the border—are regarded as poor relations. If there should at any time appear to be a departure from the strict correlation between the percentage of population and percentage of MRC money spent in a given area, I suggest that the remedy lies with Dr Hamilton and others who may think with him, to produce research and other programmes which are judged to be well worth supporting from the limited funds which are available to the various grant-giving bodies.

I am not an adviser to the MRC, nor has my view on the distribution of their funds ever been sought, and, in common with the vast majority of research workers, I also have had programmes rejected, but one keeps trying.

Yours faithfully,

P. J. HEALD

Department of Biochemistry,
University of Strathclyde, UK

A plea for hyphens

SIR,—At the risk of sounding pedantic I want to make a plea for a return to the sound practice of writing a hyphen in compound words used adjectivally. Today's general practice of omitting the hyphen in such adjectives as 'hormone-induced' and 'temperature-sensitive' is sheer sloppiness and, more seriously, often leads to ambiguity. That almost everyone does it is no excuse. Gowers, to whom lip-service is still being paid, gives very sound advice on the matter of hyphens on page 183-84 of *The Complete Plain Words* (HMSO, London 1973). So do several English and American style manuals and dictionaries.

Yours faithfully,

J. FABER

Hubrecht Laboratory,
Utrecht,
Netherlands

news and views

The structure of transcribing chromatin

from H. R. Matthews

THE bulk of eukaryotic chromatin is organised in a repeating subunit structure. Each subunit or nucleosome contains a core of two each of the histones H2A, H2B, H3 and H4 with about 195 base pairs of DNA folded or coiled outside the histones into about $\frac{1}{5}$ of its extended, B form, length. The nucleosome can be reconstituted from DNA and histones alone and in this structure the DNA is not available for transcription, at least not by *E. coli* RNA polymerase. Tissue specific non-histone proteins can modify the DNA and histone structure to give active chromatin where the DNA is accessible to RNA polymerase (see for example Paul & Gilmour *J. molec. Biol.* **34**, 305; 1968). The same situation probably applies *in vivo* and so although the bulk of the DNA in chromatin is coiled in nucleosomes what is the structure of active DNA?

Isolation of active chromatin is an important step towards studying its structure although this road is littered with scientific retractions. The other two approaches are to look for active genes in nucleosomes and to study chromatin in the electron microscope. J. M. Gottesfeld and his colleagues have described a promising approach to the preparation of an active chromatin component which involves digestion of total chromatin with spleen DNase II followed by precipitation of inactive chromatin with $MgCl_2$. The Mg^{2+} -soluble chromatin has been found to contain nascent RNA, to include DNA sequences that are known to be transcribed, but to exclude inactive DNA sequences. This has been shown in Friend cells which can be stimulated to synthesise globin messenger RNA. Mg^{2+} -soluble chromatin from unstimulated Friend cells does not contain globin genes whereas Mg^{2+} -soluble chromatin from the stimulated cells does. Moreover, in a range of cell types the amount of Mg^{2+} -soluble chromatin correlates with the overall transcriptional activity. The Mg^{2+} -soluble chromatin displays the same DNA

repeat length, 198 base pairs, as bulk chromatin which implies that the DNA repeat is a property of a class of chromatin structures rather than of a unique structure. However, complete nucleosomes from the Mg^{2+} -soluble chromatin sediment faster than nucleosomes from the bulk chromatin. Mg^{2+} -soluble nucleosomes contain RNA and non-histone proteins as well as histones and are particularly sensitive to pancreatic DNase I. Preliminary experiments suggest that Mg^{2+} -soluble nucleosomes contain regions of unstacked DNA, unlike the bulk nucleosomes (Gottesfeld, Royal Society Discussion Meeting, 1977). Under the action of RNase the Mg^{2+} -soluble nucleosomes change into the bulk nucleosome structure so Gottesfeld's results are interpreted to mean that active chromatin is in a different structure from bulk chromatin but that the active structure readily changes to the bulk structure.

In the second approach bulk nucleosomes are prepared and their DNA is hybridised to cellular mRNAs to try to detect active genes. Considerable hybridisation is observed suggesting that active DNA is indeed in the bulk nucleosome structure, but these experiments are also open to the alternative explanation that in the cell population only some copies of the genes tested are active at a given time and the hybridisation could be due to nucleosomes released from inactive sequences. Reeves (*Science* **194**, 529; 1976) suggests this may be at least partly the case from his data on the proportion of rRNA genes found in bulk nucleosomes from cells with different rates of rRNA synthesis. As the table shows, rRNA genes are under-represented in nucleosomes from cells where rRNA synthesis is high.

The rRNA genes are relatively easy

to study because they are normally highly active and reiterated but in the past few years the availability of pure eukaryote mRNAs and reverse transcriptase (RNA-dependent DNA polymerase) has allowed studies of genes such as those for globin and ovalbumin which occur in only one or a few copies per genome. These genes are also found in bulk DNA from nucleosomes so they also exist in the nucleosome structure or in a closely related structure.

Bulk nucleosomes are prepared with micrococcal nuclease which produces double-strand breaks in DNA. Another nuclease, DNase I, produces single strand breaks at 10 base-pair intervals in DNA in chromatin to give products that are of single strand lengths 20, 30, 40 . . . 160 or more bases when analysed under denaturing conditions. Weintraub and Groudine (*Science* **193**, 848; 1976) and Garel and Axel (*Proc. natn. Acad. Sci. U.S.A.* **73**, 3966; 1976) have studied the digestion of the globin and ovalbumin genes respectively. The globin gene is highly sensitive to digestion by DNase I in adult erythrocytes but in fibroblasts where it is inactive the globin gene is less sensitive, as is the bulk of the DNA. The differential sensitivity was confirmed in different cells for the ovalbumin gene. DNA complementary to mRNA was used as a probe for the presence of ovalbumin genes in DNase I digests of liver nuclei where the ovalbumin gene is not expressed, and in DNase I digests of oviduct nuclei where about 75% of the ovalbumin genes are active. These important experiments show that active genes are in a different structure from the bulk of the DNA and from inactive genes although other data suggest the 'active' structure must be closely related to the bulk nucleosome structure.

<i>Xenopus laevis</i> cells	Complement of rRNA genes	Rate of rRNA synthesis per gene	% rRNA genes in bulk nucleosome DNA
Adult erythrocytes	+/+	Minimal	86
Log phase embryonic tissue culture cells	+ / +	Normal	72
Stage 46 larvae	0 / +	Maximal	60

Electron microscopists were involved at the very beginning of the discovery of nucleosomes. What can they tell us about the structure of active chromatin? Chromatin can be seen after spreading by the Miller technique and the nucleosome structure survives although some stretching of the DNA between nucleosome cores can occur. In chromatin from nucleoli that are actively synthesising rRNA the nascent RNA molecules are packed very closely together in association with spheres of 10 nm diameter along the DNA. Are these spheres RNA polymerase complexes or nucleosomes? W. W. Francke's group (Scheer *et al. Chromosoma* **60**, 147; 1977) have measured the length of the transcription unit in pictures of active rDNA and compared it with the length of the largest RNA product that can be isolated. If this is the primary transcript then the comparison shows that active rDNA must be extended in the B form and not packed into nucleosomes. A further correlation has been noted between the total rDNA repeat as measured along active chromatin and

as determined by restriction nuclease analysis, which implies that the non-transcribed spacer is also not in nucleosomes. These are very striking results but some other electron microscopists, notably McKnight and Miller (ICN-UCLA Symposium on Molecular Human Cytogenetics, 1977) have electron microscope data which they interpret as showing the presence of nucleosomes on active chromatin. More work is needed to resolve the apparent disagreement. The electron microscope appearance of active chromatin may depend on how closely packed the RNA polymerase complexes are along the DNA or, if the active structure is easily converted to the bulk nucleosome structure and *vice versa*, then the living state may be a dynamic equilibrium which can be 'frozen' in different ways by different techniques.

Study of the structure of active chromatin is clearly at a very early stage but it is already fascinating to piece together the data from different approaches and we can look forward to more exciting progress very soon.

More water for arid lands

from Robert M. May

If the Earth were stationary, with the Sun circling round it, the global patterns of wind and weather would be simpler. There would be two convection cells, one in each hemisphere, with warm air rising at the equator, moving toward the poles, and then completing the convection pattern by returning at low elevations back toward the equator. The Earth's rotation introduces many complications, and broadly speaking the upshot is three convection cells in each hemisphere: in the first, air rises around the equator and falls back around latitude 30° N and S; the second cell, at intermediate latitudes, joins on, with poleward surface convection; the third cell has cold air falling at the pole and surface movement toward the equator. The interface between the second and third cells is relatively unstable, which contributes to the vagaries of the weather at high latitudes. These broad patterns in air circulation, and related patterns in ocean circulation, have had much influence on human history. (For example, they explain why it took so long for sailing ships to find the east coast of Australia, which was obviously a fateful event.)

As the air rises at the equator, it

expands and cools. In the process, water vapour is "squeezed out" (hence the cloud cover at the equator), and the air convected back to the surface at around 30° N and S latitudes tends to be dry. Mainly as a consequence of this fact, all the world's major deserts lie around $\pm 30^\circ$: in the south, the Kalahari, the Atacama (in Chile), western and central Australia; in the north the Sahara, the Arabian, Gobi, and Punjab deserts, and the Great Basin in the US southwest. This feature shows up in biogeographical patterns. Thus in De Candolle's classification of plant types, the smooth continuum from tropical (megathermic plants) to temperate (mesothermic), boreal (microthermic), and arctic (hekistothermic) zones is interrupted around $\pm 30^\circ$ by the band of xerophilic, or "dry-loving" plants.

As human populations grow, these arid regions pose increasingly difficult problems. Although the ultimate cause is not agreed, many of the world's sand deserts seem to be enlarging, and droughts are contributing to the economic devastation of whole nations. The six drought-stricken Sahelian nations probably provide the best known and most extreme illustration. Against this backdrop, the UN Water Conference met at Mar del Plata, Argentina, in early March (see for

example the review articles in the *Sunday NY Times*, sect. 4, page 6, 13 March; 1977). An earlier report of a panel set up by the US National Academy of Sciences, *More Water for Arid Lands* (NAS, Washington, DC; 1974), gives an excellent survey of water practices, both very new and very old, which enable the agricultural potential of arid lands to be realised.

Ancient solutions

Ancient desert dwellers collected rain by redirecting water running down hill slopes into fields or cisterns. Such agriculture was developed almost 4,000 years ago, and in the Middle East it permitted crop production on lands receiving as little as 100 mm average annual rainfall, thus sustaining agricultural civilisations in desert regions that today support only a small human population and produce few crops. In the Negev, traces of the Nabatean inhabitants remain in the rock walls they built along contours of hills to convey rainwater to lower lying fields, and in the gravel they cleared (piling it in mounds or strips) in order to bare the loess-like soils that form a crust when wet, increasing run-off. Modern versions of this latter technique use silicones, latexes, asphalt and wax to help make soils water-repellent and promote run-off. Contemporary estimates are that rainwater harvesting is possible in areas with as little as 50–80 mm average annual rainfall, although a water-harvesting catchment in Israel yielded a usable run-off in a year with only 24 mm of rain.

A variant form of rainwater harvesting is to build terraces or low walls on floodplains in order to catch and spread the flash floods that result from short, intense storms. These and other techniques have been developed by biologists and archaeologists at the Wadi Mashash, a run-off agriculture training centre in the Negev that serves trainees from arid lands all over the world. A more full account is given by Evenari *et al.* in *The Negev: The Challenge of a Desert* (Harvard University Press, 1971).

About 3,000 years ago the Persians dug qanats to bring mountain groundwater to arid plains. A qanat, an ancient Semitic word that is ancestor to "canal", is a gently sloping horizontal tunnel that brings underground water from the higher elevations of an alluvial fan down to the lower parts of the surface; a series of vertical shafts between the ground surface and the tunnel characterise the construction of a qanat, and can be used as wells and for maintenance. The old Persians built qanats on a scale rivalling the great Roman aqueducts. Many are still used, especially in Afghanistan and in Iran,

where some 40,000 qanats comprise more than 270,000 km of underground channels supplying 35% of the country's water.

Modern alternatives

Beneath many of the world's deserts are reserves of saline water, which offer possibilities for the irrigation of suitably resistant crops. If leaching and drainage are adequate, water with total dissolved salts in the range 500–1,500 milligrams per litre can be used on all but the most salt-sensitive plants. Water with up to 3,000–5,000 mg l⁻¹ can be used with highly tolerant crops. The most salt-tolerant crop studied so far is Suwanee Bermuda grass, *Cynodon dactylon*, which can tolerate about 12,000 mg l⁻¹. This is still a long way from seawater, which has a total salt content of about 35,000 mg l⁻¹.

Reservoirs and canals in arid lands are subject to heavy evaporation losses. As a proportion of total water stored, such losses involve a surface-to-volume effect, and tend to be relatively most severe for large, shallow reservoirs and ponds. Increasingly ingenious methods are directed at covering the water surface with a barrier that inhibits evaporation: these embrace liquid chemicals, usually based on long slender molecules; waxes that melt in the sun to form a film; or solid blocks of poly-

styrene or other light material. Large reservoirs, lakes, and rivers are still beyond the reach of modern technology because it is very difficult for the evaporation-suppressing system to survive heavy winds, storms and floods.

Evaporation from the soil surface of irrigated crop systems can be similarly reduced by latex, asphalt, oil, gravel, or shredded plastic mulches. Alternatively, modern "trickle irrigation" methods apply small amounts of water at frequent intervals, to a specific site near each plant. Such techniques have proved most effective for permanent tree and vine crops, and for crops grown totally under cover. Trickle irrigation will benefit from technological advances that lower costs and increase reliability and freedom from clogging. Loss of moisture by percolation in sandy soils is a problem that can be ameliorated by producing artificial underground barriers of asphalt or plastic; again the difficulty is the cost.

In a typical plant, only 1% of the water absorbed by the roots is incorporated into the plant cells; the remaining 99% is transpired back into the atmosphere. In selecting and breeding crops suitable for arid lands, clearly one of the major goals is to minimise transpiration. But the choice of appropriate plants is another story (see for example *Nature* **265**, 209; 1977). □

Vaccination against virus-induced tumours

from Robin Weiss

VACCINATION against Marek's disease (MD), a lymphoma of chickens, is one of the major success stories in viral oncology. It has aroused interest in the possibility of human vaccination against Epstein-Barr virus (a more dubious matter, I think). Marek's disease is caused by a highly contagious herpesvirus present in the feather-dust (dander) of chickens, and the acute form of the disease used to cause serious commercial loss to the broiler industry. Immunisation of one-day-old chickens with attenuated MD virus or with a related, non-pathogenic virus of turkeys protects the chickens from the disease (*Purchase Cancer Res.* **36**, 696; 1976). Precisely how the vaccine strains confer resistance to MD is not understood, and two classes of antigens, virus-specific and tumour-specific, have been recognised. The Marek's tumour-specific antigens (MATSA) are

present on the surface of lymphoma cells, but not on MD virus-infected chick kidney cells which express viral structural antigens.

Inoculation of either killed tumour cells (Powell *Nature* **257**, 684; 1975) or live, non-transformed, MD-infected kidney cells (Lesnick & Ross *Int. J. Cancer* **16**, 153; 1975) immunises chicks against MD. At the recent symposium on 'Viruses and Cancer' held by the British Association for Cancer Research at Cambridge, Powell reported that he could dissociate anti-tumour immunity and anti-viral immunity to MD by different vaccination procedures. Immunisation of chickens with glutaraldehyde-fixed MD-infected kidney cells elicited humoral antibodies that neutralise the virus, whereas immunisation with fixed lymphoma cells elicited a cell-mediated anti-tumour cell cytotoxicity that does not affect viral replication. Both types of immunisation exerted a protective effect against the development of Marek's lymphomas.

Immunity to tumours caused by

RNA oncoviruses may also be accomplished by either anti-viral or anti-tumour cell activities, and there is much interest in preparing vaccines against feline leukaemia virus (FeLV) and its associated diseases. As with the Marek's disease MATSA antigen, FeLV-induced lymphomas express a cell surface antigen, FOCMA, which appears to be unrelated to the viral envelope glycoprotein, gp70 (Stephenson *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 1219; 1977). Most of the effort in developing FeLV vaccines involves the use of FOCMA as the immunogen.

Recently however, de Noronha *et al.* (*Nature* **267**, 54; 1977) report that cats can be protected from sarcomas induced by feline sarcoma virus (FeSV) when passively immunised with antibodies directed against viral envelope antigens. FeSV is closely related to FeLV and has the advantage for experimental studies of producing sarcomas that are lethal within 4 to 8 weeks of virus inoculation, according to dose. The antibodies were prepared in goats and reacted with either FeLV or murine Friend virus gp70. They neutralise FeLV and FeSV, and are cytotoxic to FeLV-producing cells. By inoculating cats with the goat antibodies after FeSV infection, the development of sarcomas was prevented or those that had already appeared regressed. Schäfer and his colleagues (*Bibliotheca Haematol.* **43**, 86; 1975) have previously shown similar passive protection against Friend disease in mice treated with anti-gp70 serum. They propose that antibody-therapy could be widely applied to virus-induced malignancies.

One point usually ignored by investigators of oncovirus immunisation is that the tumours induced by the 'strongly-transforming' viruses grow mainly by infecting and transforming new cells rather than by proliferation of tumour cells. The importance of tumour growth by cell recruitment was clearly demonstrated by Pontén (*Natn. Cancer Inst. Monogr.* **17**, 131; 1974) for Rous sarcoma in chickens, and it is also important for the development of Friend spleen foci and of sarcomas induced by FeSV and murine sarcoma virus (MSV). Indeed, spreading infection is necessary even in cultures of non-established embryonic fibroblasts for the appearance of MSV-transformed foci (Hartley & Rowe *Proc. natn. Acad. Sci. U.S.A.* **55**, 780; 1966). While the prevention of tumour growth by antiserum to gp70 might be mediated through a cytotoxic effect on the tumour cells, an alternative mechanism may be through neutralisation of virus particles.

Linna *et al.* (in *Tumor virus infec-*

tions and immunity (eds Crowell R. L., Friedman R., & Prier J. E.), University Park Press, Baltimore, 1976)) have shown by using a complex induction system of Rous sarcomas which demands virus rescue from virogenic, non-producing cells, that neo-hatching thymectomy increases the number of sarcomas, whereas bursectomy enhances their rate of growth without affecting tumour frequency. The effects of thymectomy and bursectomy on low-dose sarcoma virus inoculation might prove more illuminating for distinguishing between anti-virus and anti-tumour cell immunity.

With the weakly-transforming oncoviruses, such as the avian lymphoid leukaemia viruses or the murine AKR-Gross virus, tumour growth seems to be largely a consequence of proliferation of one or a few clones of leukaemic cells rather than progressive recruitment. In this situation, once the malignant clone has arisen, virus neutralisation should have no role in tumour control. However, anti-rival antibodies may be important after infection yet before malignant trans-

formation, as they clearly are in natural infection of chickens and cats. Even in mice, which carry the genomes of leukaemogenic viruses in all their cells as endogenous genetic elements, only those strains that produce re-infecting virus, such as AKR and C58, have a high incidence of lymphoma. Thus a humoral response against the virus may be efficacious in preventing lymphoma. Many strains of mice naturally develop humoral antibodies, and a recent paper by Lee, Ihle, and Huebner (*Proc. natn. Acad. Sci. U.S.A.* **74**, 343; 1977) shows that formalin-inactivated virus is suitable for eliciting antibodies to gp70 and p15E, the virus envelope proteins. It will be interesting to see whether such virus-immunised mice are protected against tumours and whether cell cytotoxicity or virus inactivation plays the more important part.

I suspect that both types of immunity will be significant. Their effects may well be synergistic, both in Marek's disease, and in oncovirus-induced leukaemias and sarcomas in chickens, cats and mice. □

smoothly shifts the peak absorption wavelength. The theme of this lecture was in effect the advent of optical absorption engineering, just as Wilson had described the engineering of energy gaps.

A. D. Yoffe (University of Cambridge) spoke about solids with one- or two-dimensional order, with special emphasis on electrical conduction. The tetracyanoplatinates featured again as variants of the chain conductor, but pride of place went to the polymer (SN)_x made by polymerising S₂N₂. This is the first known polymeric superconductor, with $T_c=0.3$ K; however, at least one pair of long-chain eyebrows was raised in scepticism as to the polymeric status of this material. Attempts are being made to substitute Se for S and As or P for N with a view to improving the superconducting performance. Yoffe went on to discuss MX₂ layer compounds, a large family with M=Ti, Zr, Hf, V, Nb, Ta, Mo or Nb and X=S, Se or Te. These range from insulators through semiconductors to metals and show several kinds of order. Yoffe spent some time on Overhauser's notion of charge density waves which are well exemplified by some of these compounds. The CDW are linked with periodic displacements of atoms in the layers and in turn effect conductivity changes at the critical temperatures. Intercalation of metal atoms, such as K, between the layers can transform a metallic compound into a semiconductor or *vice versa*, and some intercalates turn out to be hard superconductors. There are prospects of developing some of these intercalates as superionic conductors for advanced batteries, and other applications (to magnetometers and as catalysts) were discussed. Much of this work stems from Yoffe's own group: research at the Cavendish on electrical conductivity of solids now spans the whole spectrum from highly ordered crystals to glasses.

A. R. Ubbelohde (Imperial College, London) demonstrated his enthusiasm for highly controlled pyrolytic carbon, especially the highly oriented, stress-annealed variety: collectively he denoted these materials as "pedigree pyrocarbons". He speculated about the band structure of this class of materials and, by extension, their potential as catalysts.

J. B. Goodenough (newly arrived in Oxford from MIT) spoke with great persuasiveness about the challenges facing the materials expert in the forthcoming burst of research on solar energy. He took his audience on a whirlwind tour of optically designed coated glass cover plates and microgrids for solar heaters, zeolites which show promise as adaptable water absorbers/desorbers for solar air-conditioning

Solid chemical physics

from Robert W. Cahn

A meeting on the Chemical Aspects of Materials Research was held in London on 21–22 March, 1977 under the sponsorship of the Chemistry Committee of the Science Research Council.

THE explicit aim of the meeting was "to stimulate greater activity amongst members of the present chemical community in certain areas of solid state science"—a praiseworthy purpose since in the British chemical family solid-state chemistry has been a poor relation, which some chemists are half-minded to show the door as an imposter. The word "materials" in the title was no doubt intended to stress the fact that this ill-defined branch of chemistry generates its fair share of industrial utility. The title of this report is as accurate a representation as any of what the symposium was actually about; "solid-state chemistry" would have been appropriate had there been more emphasis on reactions. Fuzziness at the borders of disciplines is a symptom of healthy activity: the SRC inevitably sometimes runs into demarcation problems, but makes amends for the occasional administra-

tive *longueur* by its sponsorship of genuinely interdisciplinary meetings such as the one under review.

The meeting consisted of ten hour-long lectures (a few of which are reported below) interspersed by generous discussion time, nowadays a highly untypical format. The value of the symposium was enhanced by the fact that speakers were able to follow their subjects in depth and also follow side-tracks.

B. L. Wilson (Plessey) opened the proceedings with a survey of the 'engineering' of highly alloyed semiconductors with adjustable bandgaps: (Ga, In, Al) (As, P). P. Day (Oxford) concentrated on unusual optical and magnetic phenomena, in particular ionic crystals in which optical absorption can be sharply altered by an applied magnetic field. FeCl₂ is the prototype: separate, ferromagnetically-bonded layers are coupled antiferromagnetically, and the anomalies arise when the layers become magnetically saturated and the salt effectively breaks down into two microphases, nucleation being catalysed by crystal dislocations. Other salts (RbCrCl₄, for example) have, at certain wavelengths, selective peak absorption, the magnitude of which is sharply dependent on magnetisation. In a third category, the tetracyanoplatinates, ionic substitution

(potentially a major application), and the conceptually difficult spectrum of phenomena ranging from straight electrolysis, through electrically assisted photolysis, to straight photolysis of water at electrically connected semiconducting electrodes without benefit of external batteries. (This whole field was initiated by a Japanese paper in *Nature* in 1972.) TiO_2 and SrTiO_3 have been tried, but other semiconductors with smaller bandgaps need to be developed, perhaps with dye sensitisers incorporated. The potential importance of this technology is very great.

In a beautifully lucid lecture G. Allen (Imperial College), basing himself on the Flory-Huggins insights, explained just why useful two-phase polymers can in general only be made by the stratagem of block copolymerisation, which alone permits the correct balance between the tendency to phase separate and the physical impossibility of doing so completely. To a metallurgist used to the interpretation of metallurgical phase diagrams in terms of the configurational entropy of crystalline solid solutions, the rationalisation of polymeric phase diagrams in terms of the configurational entropy of polymer chains was convincing. The laws of mixtures governing the physical properties of such systems were exemplified and the prospects for ungrafted solution-blended two-phase systems outlined. (Physically mixed polymer systems apparently have a Houdini-like proclivity for escaping from their most elaborate entanglements.) The formation of networks from T-shaped monomers was explained. This approach permits the design of tough structural plastics and rubbers, of materials with controlled water permeability and other special-function polymers. In this lecture the rationale of a lifetime's research strategy was made manifest.

The symposium was an unqualified success, and it is to be hoped that the SRC will feel encouraged to continue to sponsor such occasions. □

Green light for the signal hypothesis?

from Pamela Hamlyn

THE work on secreted proteins, particularly those of the liver and pancreas, has demonstrated that these proteins are synthesised on ribosomes bound to endoplasmic reticulum, then transferred through the membrane into the lumen so that their synthesis and secretion form a continuous process. What

is much less certain is the way in which the messenger RNAs which code for secretory proteins are selected for translation by membrane-bound ribosomes. That the selection may be a consequence of a particular nucleotide sequence in these mRNAs, or due to the activity of a separate class of ribosomes, are considerations which have found some experimental support, but the suggestion for which there is most experimental evidence is that it is a characteristic of the protein itself (namely the amino acids found at the amino terminus of a precursor form of the mature protein) which leads to its synthesis by membrane-bound ribosomes. A model for the mechanism by which the ribosome is brought in contact with the membrane by way of the N-terminal amino acids has been suggested by G. Blobel and his colleagues (for example, Blobel & Dobberstein *J. cell Biol.* **67**, 835; 1975).

Some very convincing evidence that it is the protein product, rather than the mRNA or ribosomes, which is responsible for the specificity of attachment of polysomes to membranes, has been produced by Harvey Lodish's group at the Massachusetts Institute of Technology (Wirth *et al. Cell* **10**, 253; 1977). Working with Sindbis virus-infected chicken embryo fibroblasts, they have isolated a 26S viral mRNA which codes for three proteins; one is a core protein which is eventually associated with the 42S RNA viral genome and the other two are glycoproteins required for the lipid bilayer which envelops the mature virus. The proteins are translated sequentially from one initiation site on the mRNA: first the core protein, then the two envelope proteins. This recent report is concerned with the localisation of the polysomes involved in the synthesis of these two classes of protein. It was found that 70% of the 26S RNA is bound to membrane and that newly synthesised core protein is localised on the cytoplasmic side of the endoplasmic reticulum and remains free in the cytoplasm, whereas the envelope proteins remain associated with membrane. Since the same ribosomes translate the same mRNA this is very persuasive evidence that neither the ribosome nor the mRNA determines which proteins are synthesised on membrane. In the model described by Wirth *et al.* it is proposed that at the beginning of translation the 26S RNA is free in the cytoplasm whereas the core protein is synthesised and released. As translation of the envelope protein proceeds, its amino-terminal sequence initiates an interaction with the membrane leading to the ribosome becoming 'membrane-bound'. No amino acid data is available

for the synthesised proteins but it has been observed that the first of the two glycoproteins is produced in a larger form than is found in the lipid envelope.

The light chain of mouse immunoglobulin has been known for some time to be synthesised in a larger form than is eventually secreted. This has been shown to be due to about 20 extra amino acids preceding the amino terminus of the mature protein. Israel Schechter and his coworkers have been working on the sequence of the amino acids in what they call the 'extra piece' in several different light chains (Burstein & Schechter *Proc. natn. Acad. Sci. U.S.A.* **74**, 716; 1977). Two completely sequenced, and other partially sequenced, 'extra pieces' reveal that although these regions are not particularly homologous in sequence they all contain a very high proportion of hydrophobic amino acids. This fits nicely with the suggestion that this region is responsible for the interaction with endoplasmic reticulum leading to the ribosomes becoming bound to membrane. However, Burstein and Schechter envisage that the hydrophobic region would also allow the protein to associate with other membranes and suggest in particular that it may interact with the cell surface membrane to serve as an antigen-recognising receptor. This hypothesis can be tested experimentally: it will be interesting to see if the 'extra piece' is involved in several different protein-membrane interactions. □

Iranian natural history

from Peter D. Moore

THE past 30 years have seen an enormous expansion in amateur interest in natural history in Europe and North America. This surge has been accompanied and encouraged by a parallel proliferation of field guides for the identification of such organisms as birds, plants and butterflies. Taxonomy apart, these developments have been of great benefit to the serious study of distribution patterns, behaviour and migration. In many other parts of the world, where documentation is at present far less thorough, the galvanisation of the potential mass of amateurs may simply be awaiting the provision of accessible field guides to identification.

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In Iran, the government Department of the Environment regards such provision as an urgent priority. It began its programme of publishing field guides in 1975 with the production of *The Birds of Iran* (eds Scott, D. A., Hamadani, H. M., & Mirhosseini, A. A.). Many difficulties were faced in the production of that book. The choice of language (the book is written in Persian) the names used for birds in the country (selection from many local names had to be made) and the art work (illustrations from British and French field guides had to be used due to a lack of suitable indigenous artists) all presented problems. The book which resulted is impressive in its scope and the distribution maps are useful even to the non-Persian speaking visitor, if he is prepared to learn and use arabic numerals.

This year has seen the publication by the Iranian Department of the Environment of *A Guide to the Mammals of Iran* (ed. Harrington, F. A.). This time the text is in English and the art work, which has been splendidly executed, was specifically commissioned and was undertaken by R. S. Robinson. All 148 mammals recorded from Iran, including the tiger and the lion which are now probably extinct there, are described and 112 are illustrated. Iran covers a large range of geographical zones in its span between the Gulf and the Caspian and the arrangement of the book is particularly helpful in grouping species into habitat sections rather than using a strictly taxonomic order. In this respect the *Guide* innovates in a manner which may well be emulated in future western publications in this vein. Harrington's broad experience of the Iranian fauna is evident in the text and his work will undoubtedly pave the way for further studies, particularly on detailed geographical distribution in the country. Work is now underway on the next volume in the series which will deal with reptiles.

The difficulties and the needs of the botanist in Iran are rather different. The high central plateau is rich in endemics and the flora is therefore extremely large. A definitive *Flora Iranica* is still in the course of production, being edited and compiled by K. A. Rochinger at Vienna. At present, therefore, the production of popular field guides must await the full description of the Iranian flora, and new species are constantly being described. A higher priority has been the provision of a scientific publication in which the gradually unfolding Persian taxonomic discoveries can be communicated. In the past, most descriptions of new species, local floristic lists and ecological work have been

published in western journals; much taxonomic work has found its way into the *Notes of the Royal Botanical Garden, Edinburgh* for example. Now a new journal has been launched—the *Iranian Journal of Botany*—edited at the Ariamehr Botanical Garden in Tehran. It is evident from the first issue that this will be a most useful source of reference to all who are interested in the flora of the Middle East. Wendelbo provides an annotated check list of Iranian ferns; Eckblad summarised the scant information available concerning the gasteromycetes of Iran; biogeographical implications of newly described species are discussed and a floral list from the important Kavir Protected Region is included. The journal is in English and is lavishly illustrated with colour photographs; it is currently only available directly from the Ariamehr Botanical Gardens.

These efforts of the Iranians to distribute all available information must be highly commended. It must now be seen whether, as in the west, this dissemination results in the encouragement of further work. □

New discontinuity in the Atlantic

from M. Whitfield

ALTHOUGH, from the surface, the oceans may appear as remarkably homogeneous and featureless bodies of water they are in fact made up of an intricate three-dimensional patchwork of individual water masses. These water masses are layered according to their densities which in turn are defined by the salinity (S) and potential or *in situ* temperature (θ) of the component waters. The water masses are therefore most simply identified as clumps of points on a θ/S plot and the relationship between the nutrient (silicate, nitrate, phosphate) and oxygen concentrations and S or θ provide useful additional chemical fingerprints. One of the important tasks of physical and chemical oceanographers is to identify the origins of these bodies of water, to trace their movement through the oceans and to document their eventual decay by mixing with adjacent water masses. Such information is crucial to our understanding of oceanic mixing processes in general and in particular will affect our interpretation of the dispersion of man-made contaminants throughout the oceans.

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A recent paper by Broecker *et al.* (*Deep-Sea Res.* 23, 1083; 1976) presents the first results of an unusually detailed analysis of water masses throughout the Atlantic and illustrates some of the difficulties involved in unravelling these mixing patterns. They use results from the well-documented GEOSECS cruises which confirm many of the long established features of the Atlantic circulation pattern (Fig. 1). Their data indicate however that the boundary between the North Atlantic Deep Water (NADW, Fig. 1) and the Antarctic Bottom Water (AABW, Fig. 1), as indicated on θ/S plots, is far too sharp to be the result of vertical mixing between these two water bodies. They suggest that yet another water body is sandwiched between these two layers. This water is characterised by a potential temperature of 2 °C (hence the name Two Degree Discontinuity Water, TDDW) and a salinity of 34.9‰ (Fig. 1). Water with these characteristics can be generated by mixing water from the North Atlantic, (Northern Component Water, NCW; 8 parts) with colder, less saline water from the Southern Atlantic (Southern Component Water, SCW; 1 part) which has a higher oxygen content and a lower silica content. TDDW can be traced throughout the Central Atlantic (from 35°N to 33°S) at depths from 4,000 m in the north to 3,200 m in the south. The concentrations of nutrients (phosphate, nitrate and silicate) and oxygen of TDDW in the western basins are consistent with the 8:1 mixing ratio. As the TDDW is traced towards the eastern basin the nutrient and oxygen patterns shift in a highly correlated



A hundred years ago

FINNISH papers report that vast masses of smoke are issuing from a mountain adjoining the river Tana, and that the snow in the vicinity has been melted away. The region has hitherto been free from evidences of volcanic activity. The theory has often been advanced that the gradual elevation of the shores of the Gulf of Bothnia is due to volcanic forces, and it is possible that these are finally seeking a vent.

The royal tigress in the Berlin Zoological Gardens, lately brought forth a litter of two, which she utterly refused to take care of. They were accordingly placed amidst the family of a Newfoundland dog, who welcomed the newcomers warmly and bestows upon them all necessary maternal attentions. From *Nature* 16, 17 May, 54; 1877.

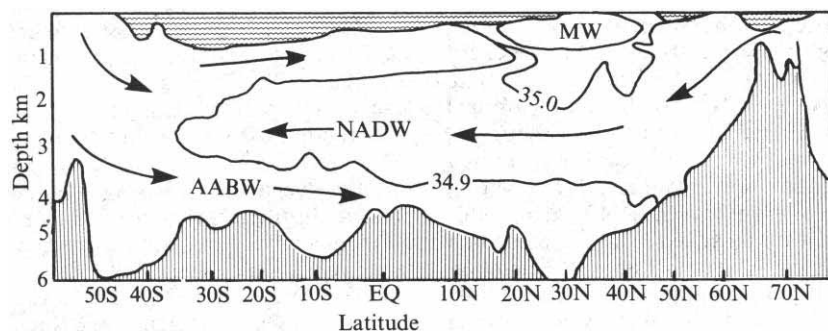


Fig. 1 Basic features of deep water movement in the Atlantic ocean as shown by a GEOSECS track down the centre of the western basin (adapted from Fig. 2 of Broecker *et al. op. cit.*). North Atlantic Deep Water (NADW) originating from the Labrador, Greenland and Norwegian Seas, flows south to split a northerly flow of Antarctic water. The Antarctic Bottom Water (AABW) results from the mixing of Weddell sea water and deep circumpolar water. A warm saline tongue of Mediterranean water (MW) intruding from the east interrupts the northerly flow of the upper layer of Antarctic water. Horizontal shading indicates the approximate extent of basal thermocline waters.

pattern that is consistent with the release of nutrients, originally locked up in biological detritus, by respiration and silica dissolution. It seems likely that upwelling areas off the west coast of Africa provide the main source of this organic debris so that nutrient levels rise and oxygen levels consequently fall on traversing from west to east.

Any hypothesis concerning the origin of TDDW must allow water masses from both ends of the Atlantic to be blended and spread over the whole central region. The rate of spread must be rapid enough to maintain the sharpness of the two degree discontinuity (TDD) against erosion by vertical mixing but not so rapid that the nutrient composition is homogenised throughout the layer. It is a measure of the difficulties inherent in understanding oceanic mixing processes that no convincing or consistent explanation can be given at this time for such a widespread feature.

A steady-state hypothesis proposed by Broecker *et al.* is based on the confinement of deep water movements by the Rio Grande Rise (at approximately 30°S) and suggests that TDDW is generated in the southern half of the Atlantic. This is inconsistent with the fact that the contribution of SCW to TDDW appears to decrease, rather than increase towards the south. It is also difficult to reconcile with observations that a young variety of TDDW (high in ¹⁴C and low in nutrients) is apparently forming just north of the 30°N boundary in the western basin. The alternative is to shed the concept of steady state mixing, which has so far formed the foundation of oceanic mixing models, and to assume that TDDW is a residual feature from an earlier stage in the ocean's history when the intrusion of AABW was much more extensive than it is now. The TDD, as we see it now, represents the cutting edge

of a more recent influx of NADW as it erodes away the remnants of this earlier intrusion from the south. Unfortunately the oxygen concentrations and the ¹⁴C ages of the various water bodies do not support the idea that water in the mixing zone is any older than the over-riding NADW. The mystery over the origin of TDDW is compounded by the fact that inflections in the θ and S against depth profiles are invariably several hundred metres deeper than the TDD in the western basin. This observation, which is inconsistent with the traditional physical picture of vertical mixing invoked in both the steady state and erosional models mentioned above, may provide a vital clue to the origin of this sharply defined and widespread feature.

Molecular genetics at Hirschhorn

by Eleanor Lawrence

The Third EMBO Annual Symposium, entitled Molecular Aspects of Gene Function, was held at Hirschhorn, West Germany, on 26–30 April 1977.

THE molecular biology of gene expression provided an umbrella large enough to shelter topics as diverse as bacterial chemotaxis, how the silkworm lays down its eggshell, and the structure of the cell's microfilament skeleton. But in the mainstream of molecular genetics the most rapid advance is undoubtedly in the dissection of the fine structure of the eukaryotic genome, thanks to the advent of recombinant DNA technology and fast sequencing methods. On the broader front, re-

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combinant DNA techniques promise to transform the study of the genetic basis of differentiation and development by bridging the gap between the biochemistry and classical genetic analysis.

At the level of the gene the hunt is now on for the elusive eukaryotic regulatory sequences. The complete sequence of the yeast 5S rRNA gene and its immediate flanking sequences has been determined by W. Gilbert and colleagues (Harvard University). Various interesting features appear in the region immediately preceding the transcribed portion but as yet the sequence alone can give few clues to the RNA polymerase recognition site, and there are no homologies with the well-known palindromic prokaryote promoter sequences. The gene is bounded at both ends by an oligo (dT-dA) tract. An oligo (dA-dT)-rich region has also been found by R. A. Flavell and A. Jeffreys (Amsterdam University) in the 5' region flanking the rabbit α -globin gene. Using globin cDNA plasmids as probes to identify globin gene sequences in rabbit DNA, they have been able to construct a restriction map of the chromosomal gene and its surrounding regions and have subjected these to preliminary analysis.

In the sea urchin histone repeat unit being sequenced by M. Birnstiel and his colleagues (University of Zurich), a partially homologous sequence is found 20 nucleotides upstream of both the histone H1 and H2A mRNA initiation sites, and another homology occurs some 100 nucleotides upstream in both genes. To determine what part if any these sequences play in gene expression Birnstiel intends to use the oocyte transcription/translation system described by John Gurdon (MRC Laboratory of Molecular Biology, Cambridge) (see *Proc. natn. Acad. Sci. U.S.A.* **74**, 1502; 1977). In principle, the promoter region could be identified by producing various DNAs containing the gene plus different lengths of flanking sequence and determining which of these are transcribed when injected into the oocyte nucleus. Birnstiel has also made some modifications to the technique which he claims make it easier to use by those inexperienced in oocyte nucleus injection—centrifugation makes the nucleus bulge out the oocyte wall leaving a neat ring of pigment granules as an injection target.

Nucleotide sequences alone have failed to betray the eukaryotic promoters; but novel and exciting techniques of DNA modification (Gilbert) can be used to determine the exact binding position of protein on DNA if the nucleotide sequence is known and may provide a direct handle on the problem. Using the phosphate-specific

reagent ethylnitrosourea, Gilbert has shown that in the *lac* repressor-operator model system the *lac* repressor binds to one side only of the DNA double helix rather than being wrapped around the DNA. The bases involved in RNA polymerase recognition are also being investigated, by examining the effects of RNA polymerase binding to *lac* DNA on the reactivity of the individual bases to modifying reagents.

Another approach to the analysis of *lac* operator-repressor binding was demonstrated by J. H. Miller (University of Geneva). He and his colleagues have used novel genetic techniques (see *J. molec. Biol.* **109**, 275, 303; 1977) to generate repressors with known amino acid changes. Among them is one with almost 10,000 times wild type affinity for operator and which also binds to non-operator DNA such as lambda and SV40. By determining whether this non-specific binding involves any particular sequences they hope to identify those features of the binding sequence which are common to all and thus identify the parts of the operator crucial for repressor recognition. Using the nonsense mutations accumulated during this work, they have also been able to determine the characteristics of one type of mutational 'hotspot'—genetic regions which are much more easily mutated by specific mutagens. They find that the hotspots preferentially mutated by 2-aminopurine and at which mutations also arise spontaneously are always part of a unique sequence which has 5-methylated cytosines in a particular position (the *i* gene has been sequenced by Gilbert and colleagues). This tie-up between methylated bases and mutability leads Miller to speculate on the wider role of methylation *in vivo*.

In connection with recognition sites on mRNA several speakers, especially L. Gold (University of Texas) reminded participants that the three-dimensional structure must be considered and not just the sequence. He illustrated this with reference to a theoretical structure for *lac* repressor mRNA.

Any discussion of eukaryotic gene expression however, has to take into account that in reality eukaryotic DNA is packaged into chromosomes in the form of chromatin. *In vitro* transcription of chromatin provides one way of investigating the possible regulatory role of the associated histone and non-histone proteins. B. O'Malley (University of Houston) reported a set of experiments on transcription of chick oviduct chromatin. Using *E. coli* RNA polymerase, and a plasmid-derived probe of ovalbumin cDNA, he showed that oestrogen-stimulated oviduct chromatin retained transcriptional specificity *in vitro*, and by reconstitution

experiments that this specificity is mediated by non-histone proteins. Although these results are not novel (Paul and Gilmour published similar data several years ago as did other groups) the low level of endogenous sequence, combined with the use of mercurated nucleotide precursors to allow isolation of newly-synthesised RNA, make this work the most convincing presented to date. However, as P. Chambon (Institut de Chimie Biologique, Strasbourg) stated in discussion this is still a controversial area of research and the proponents of chromatin reconstitution do occasionally shift their arguments to meet the latest technical advances. Perhaps the most impressive result O'Malley presented was that he could switch on ovalbumin mRNA transcription by adding the progesterone/receptor complex to isolated 'switched-off' chromatin; this natural hormone switch activated the chromatin some 10 to 50 times over the control. O'Malley is now isolating genome DNA sequences containing the ovalbumin gene by affinity chromatography on cDNA and mRNA cellulose columns, and has already obtained a purification of more than 100,000-fold. Soon he will have sufficiently pure DNA to clone the gene sequences and so be able to study the control regions near the structural gene.

The search for possible gene-specific or sequence-specific DNA-binding proteins which may play a part in regulating the expression of particular genes was illustrated in work reported from W. Gehring's laboratory (Biozentrum, Basle). Two DNA-binding proteins isolated from unfertilised *Drosophila* eggs were shown to bind specifically to two chromosomal fragments from Gehring's bank of cloned DNA fragments representing the complete *Drosophila* genome. One of these hybridises *in situ* only to the nucleolus, strongly suggesting that its corresponding DNA-binding protein is specific for ribosomal DNA sequences; the other hybridises to one band on chromosome 3.

The genetic basis of differentiation and development is now under investigation in a variety of systems. One of the most promising is that described by F. Kafatos (University of Athens) in the silkworm *Bombyx*. Its chorionic proteins which are laid down in strict sequential patterns to form the eggshell, are determined by a system of two or three multigene families whose mechanisms of regulation and expression during differentiation and development are now, with recombinant DNA technology, amenable to full analysis. *Bombyx* also obligingly provides several chorionic mutants for comparative analysis.

After determining the protein electrophoretic pattern at different stages of eggshell formation, Kafatos together with T. Maniatis and colleagues at Harvard University is now constructing cDNA plasmids representing specific chorionic mRNAs, from the heterogeneous mRNA population present during choriogenesis. Some 20 different clones have been identified so far, two of which have been found to represent proteins expressed at the same developmental stage and one at a different stage. By looking at the pulse-labelled nuclear RNA at various stages using the cDNA plasmids as probes, it will be possible to answer the question of whether the protein pattern is controlled by specific transcription or by translation of pre-existing messengers. Chromosomal DNA clones are also being constructed and will be used to determine whether evolutionarily similar chorionic protein genes are linked, to determine whether genes expressed at the same time are linked, and to study the putative regulatory sequences flanking the genes.

One interesting report (arising out of the study of the developmental genetics of the slime moulds) was R. A. Firtel's (University of California, La Jolla) description of translation of a cloned actin gene from *Dictyostelium* in *E. coli* minicells. So far his evidence consists of the appearance of a new protein, which runs in the same position as actin on one-dimensional gel electrophoresis. Confirmation of specific translation awaits the full identification of this new protein.

Now that it has become possible to look directly at the regulatory sequences surrounding the gene a great deal of attention is being focused on the transcriptional control of gene expression. But regulation also occurs at many other levels. Differential stability of mRNAs as well as differential transcription must be invoked to explain the patterns of mRNA abundance and copy complexity found in some developing systems, and R. Williamson (St Mary's Hospital Medical School, London) reviewed evidence that control is also exerted at the stage of mRNA processing from nucleus to cytoplasm. J. Bishop (University of Edinburgh) reported evidence from his laboratory that in different types of mouse cell a substantial proportion of the total mRNA sequences are common to the different cell types at a low level. Margaret Buckingham (Institut Pasteur, Paris) described changes in mRNA copy complexity and abundance in myoblast differentiation and also showed that particular mRNAs are more stable in differentiated myotube compared with myoblast cells, whereas others show no change. □

review article

Super-luminal expansion in extragalactic radio sources

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Very long baseline (VLB) investigations of compact extragalactic radio sources can often be interpreted in terms of two non-thermal components apparently separating with speeds in excess of the speed of light. Given the conventional physical laws and the cosmological interpretation of large redshifts, the available morphological evidence, while inconclusive, is most compatible with a general class of models in which a signal propagating with a speed close to that of light is scattered by an appropriately shaped screen. Possible observations are discussed which might discriminate between this and alternative explanations.

THE use of very long baseline (VLB) interferometry has made it possible to resolve hitherto inaccessible details of compact extragalactic radio sources, and seems to show that they are made up of two or more components. The original observations have now been repeated at intervals over the past five years, and have revealed that these components are in the process of moving apart. The spectacular implication of this discovery is that, if the radio sources are at the large cosmological distances suggested by their red shifts, the components must be separating at apparent transverse velocities more than twice the speed of light¹⁻³. In this article we review and assess the various theoretical explanations that have been proposed to account for this remarkable phenomenon, our main aim being to emphasise the theoretical and observational uncertainties which must be resolved before these objects can be reliably interpreted.

The projected sizes of the rapidly expanding components are typically only a few parsecs and so they are presumably linked to the primary energy source powering the associated active galactic nucleus—indeed this is one reason why VLB observations are of such great astrophysical interest. However, observations in other parts of the electromagnetic spectrum have sometimes shown even more rapid variations^{4,5}; light travel time arguments imply that the 'prime mover' may be even smaller than the radio source.

We have attempted to provide a loose kinematic classification for the models discussed in this paper as shown in Fig. 1 (although the boundaries between the different categories are rather blurred and the classification is not exhaustive). In 'intrinsic' models, the apparent expansion is directly related to the radio emission process; these models are 'causal' or 'acausal' depending on whether or not the expansion is due to one or more independent events. In 'extrinsic' models, the apparent expansion results from scattering or focussing by the intervening medium along the line of sight.

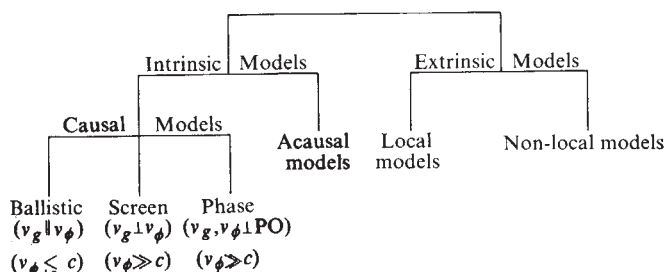
The radio emission is commonly attributed to an incoherent synchrotron-like process involving relativistic electrons (although the evidence for this is less convincing than for extended radio sources). Many of these sources have had their total

flux monitored^{6,7} for more than ten years and have long been known to be variable. In many cases, the synchrotron hypothesis can lead to a theoretical difficulty, the so-called 'inverse Compton catastrophe'^{8,9}. This can arise if the linear size of the source is limited to the distance light can travel in the time taken for the flux density to halve, thus putting a lower limit on the radiation brightness temperature and energy density and an upper limit on the magnetic energy density. When the dominant contribution to the energy density is in the form of radiation rather than magnetic field, the total energy requirements of the source then become implausibly high. This problem disappears if the sources are expanding with speeds $\sim c$ (refs 9–12). Furthermore, in most sources, the relativistic particle pressure is found to exceed the magnetic pressure⁹. Therefore, unless the relativistic plasma is bound by a much higher density of thermal plasma (and this can probably be excluded¹³ on other grounds), relativistic expansion is to be expected. So these arguments, which are independent of the observed angular structure (and were given before the VLB observations), at least support models involving intrinsic relativistic expansion which as we shall see seem most attractive on other grounds.

Dynamics of relativistic expansion

Our understanding of relativistic gas dynamics is largely derived from solutions of a few idealised problems. We mention some of these here in order to demonstrate that relativistic kinematical effects (discussed below) can be accommodated in a framework that is both dynamically and astrophysically plausible.

Fig. 1 Possible classification of the physical models discussed in the text.



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We shall consider first the free expansion of an initially uniform spherical cloud of relativistic gas. If an initially uniform cloud of ultra-relativistic plasma starts expanding from radius r_0 , then the Lorentz factor of the bulk expansion when the sphere attains a radius $r \gg r_0$ would be $\gamma \sim r/r_0$ (refs. 14–16), the material being concentrated mainly in a thin shell of thickness $\sim r_0$. In order to conserve total energy, the internal (thermal) energy per particle, measured in a co-moving frame, must be reduced by a factor $\sim r_0/r$. If the cloud is composed of electrons and protons, then the bulk Lorentz factor will saturate when the energy per particle $\sim m_p c^2$ and the characteristic electron energies will subsequently decay $\propto r^{-2/3}$ if they expand independently from the protons with a specific heat ratio of $4/3$. In some models in which the central energy source derives from ‘magnetic accretion’ on to a Kerr black hole, an electron-positron plasma may be produced¹⁷ and this could attain bulk Lorentz factors comparable with the inferred initial random Lorentz factors of the emitting electrons. Free expansion can occur either impulsively, if the surrounding medium is dynamically unimportant, or continuously in a supersonic wind. If this wind were initially subsonic, then either a nozzle geometry¹⁸ or a relativistically deep potential well¹⁹ would be needed to achieve a steady relativistic trans-sonic change. Variability could be induced in an expanding wind by changing the volume emissivity in a dynamically insignificant fashion. However, if the emission processes are synchrotron and inverse Compton radiation, the observed optically thin power will typically decay very rapidly with radius and so it should be dominated by the model-dependent conditions at small radii²⁰.

Second, we consider relativistic blast waves. If the external medium has a density $\rho > E/r^3 c^2 \gamma^2$, with E the energy of the expansion, then it cannot be ignored. A strong shock front will expand outwards with a Lorentz factor $\sim \sqrt{2}\gamma$. The swept up material will be concentrated within a shell of thickness $\sim r/\gamma^2$ and if the explosion is impulsive with the external medium uniform, $\gamma \sim (E/\rho c^2 r^3)^{1/2}$ (refs. 20–22). When r gets so large that the shock stops being relativistic then there is a smooth transition towards the ordinary Sedov²³ solution. Any realistic dynamics compatible with the VLB data must certainly involve deviations from spherical symmetry. Fortunately these results are easily generalised to non-spherical geometries as long as the expansions are at least mildly relativistic. This is because, to a first approximation, each segment of the expanding shell moves independently. A dynamical advantage of blast wave models is that the shocks can be radiative, so that the mechanical energy associated with the explosion can be transformed to radio emission with a high efficiency. This is in contrast with free or adiabatic expansions and models in which the radio emission is associated with a moving plasmoid.

Finally, we may envisage a fast-moving condensed object. The radio-emitting plasma need not be undergoing any expansion if it is coupled (either electromagnetically or by ram pressure) to a rapidly moving object. One attractive conjecture is that when a binary system comprising two condensed objects (neutron star or black hole) coalesce as a result of the radiation of gravitational waves, tidal effects become important and the individual components are broken up²⁴. One or more components of appreciable size can then be ejected relativistically²⁵. Note that in this case the expansion speeds will be constant, instead of accelerating or decelerating as in the case of free expansion of relativistic gas or relativistic blast waves.

Of course, some aspects of all three possibilities may be involved. For instance, the early phases of an outburst may resemble freely accelerating expansion, followed in the later stages by either gradual deceleration in a uniform medium or sudden retardation when a comparatively dense phase of the surrounding material is encountered.

Kinematics of causal models

When a source changes in a time comparable with its size in units of c , the kinematics of the emitted radiation must be carefully analysed and it is possible for a distant observer to

see apparent super-luminal expansion. For causal models, we imagine that an outburst occurs at a point P at a time $t = 0$, as measured by a distant observer O. A ‘signal’ such as a blast wave^{20,22} or a burst of low frequency radiation from a newly-formed pulsar^{26–28} then moves radially outwards from P. This signal may either be directly associated with the emission (for example, in the case of a radio-emitting plasmoid)^{10,11} or trigger it (for example, in the case of a low frequency electromagnetic wave that can accelerate electrons but not ions to relativistic energies, so that the electrons are held in place by the inertia of the ions)²⁷. We distinguish four speeds associated with this signal: a group velocity $v_g < c$ which is the material velocity of the signal; a phase velocity v_ϕ which is the space velocity of some directly observed feature such as a radio centroid or the outer edge of a resolved component; v_0 , the apparent velocity observed at O, normal to OP; and the actual physical velocity $v_{em} < c$ of the emitting material or of the frame in which the emissivity is approximately isotropic). In some models all four speeds are different. v_0 and v_ϕ are related by

$$v_0 = \frac{v_\phi \sin \theta}{1 - (v_\phi/c) \cos \theta} \quad (1)$$

or

$$\cos \theta_{\pm} = \frac{v_0^2/c \pm [v_\phi^2 + (v_\phi^2 v_g^2/c^2) - v_g^2]^{1/2}}{v_\phi [1 + (v_0/c)^2]} \quad (2)$$

where θ is the angle between v_ϕ and PO.

In ‘ballistic models’ of super-luminal expansion, the phase and group velocities are both approximately parallel to OP and usually approximately equal. From equation (1) we then see that $v_0 \leq v_\phi \gamma_\phi$, where equality implies that $\theta = \cot^{-1}(c/v_0)$ large observed speeds v_0 can only occur if v_ϕ , which is generally $< c$, is almost parallel to the line of sight. As an example, consider a spherical model in which $v_g = v_\phi$ is the relativistic radial velocity of a radio emitting shell. If v_g is constant, then the surface observed at a fixed observer time t is an ellipsoid with focus at P and eccentricity $v_g/c^{10,11,20,29,30}$. The observed width is the minor axis of the ellipsoid, $2\gamma_g v_g t$, ($\gamma_g = (1 - v_g^2/c^2)^{-1/2}$), and so the observed expansion speed is $v_0 = 2\gamma_g v_g$ which exceeds $2c$ when $v_g > 2^{-1/2}c$. The observed shape can be straightforwardly calculated when v_g is a function of position. If, for instance, v_g changes only with radius r the locus becomes an ovoid, the blunt end being towards O for a decelerating (and the sharp end for an accelerating) expansion. Provided that $d \ln \gamma_g / d \ln r = -n < \frac{1}{2}$, $v_0 = 2\gamma_g v_g \propto t(-n/(2n+1))$, where v_g is the group velocity of the edge of the observed disk at the retarded time. If the emitting region of the source is enlarging in this fashion then it follows that the actual source dimensions are $\sim \gamma_g$ larger than the projected size (that is, $\sim \gamma_g^2 ct$), assuming approximate spherical symmetry.

Clearly a spherically symmetric model cannot explain double structure. In a straightforward modification, two radio-emitting plasmoids are ejected along anti-parallel directions^{31,32}, the velocity of the nearer making a small angle $\sim \gamma_g^{-1}$ with PO, the super-luminal velocity being mostly attributable to the approaching plasmoid. However, this occurs with a probability $\sim (\cos \theta_+ - \cos \theta_-) \leq 2c^2/v_0^2$, which is typically very small when $v_0 \gg c$.

In ‘screen’ models, v_ϕ and v_g are approximately perpendicular and the radio emission is triggered by a relativistic signal ($v_g \sim c$) illuminating a pre-existing surface so that $v_0 \gtrsim c$. Usually v_ϕ is normal to the line of sight so that $v_0 \sim v_\phi$. A possible physical manifestation is the intersection of a relativistic blast wave with a dense plasma (or another blast wave) wherein the kinetic energy can be efficiently radiated away. Of course, with an appropriately designed screen, we have $v_\phi \gg c$ (for example, if a relativistic signal illuminates a paraboloidal screen with focus P, axis PO). For a roughly spherical screen of radius R illuminated by a concentric spherical pulse of speed c , a distant observer will see a circle of radius $\sim (2Rct)^{1/2}$

for $t \ll R/c$ that seems to expand super-luminally until the apparent radius $\sim 2^{-1}R$ (refs 33, 34). If the screen is a thin ring (for example, a rotating, gaseous disk surrounding a central gravitating object), then the observer will see an expanding double whose separation is decelerating. (Deceleration is characteristic of screen models and is generally more likely to occur than acceleration.) A rotating searchlight³⁴ forms the basis for a kinematically similar model.

The signal motion need not be rectilinear. One ingenious suggestion³⁵⁻³⁷ is that the emitting electrons are channelled at relativistic speeds along the north and south polar field lines of a magnetic dipole. They would then be visible only when they intersected the line formed by the locus of field lines tangent to OP. If the emitting particles are all generated in a single outburst then an expanding double ($v_0 = 4.4c$ (ref. 35) in the equatorial plane) will be seen aligned with the rotation axis. However, because the synchrotron emissivity falls off so rapidly ($\propto r^{-9}$ if the magnetic moment is preserved), it is difficult to account for the equality of the radio components or to evade prohibitive synchrotron losses at early times in this model.

In most types of simple screen model super-luminal effects can usually be achieved without the observer's having a privileged orientation (in contrast to ballistic models). Apparent velocities $v_0 \gg c$ will, however, only be observed for a fraction $\sim (c/v_0)^2$ of the total emission time (the duty cycle) and the actual size of the screen will typically exceed the observed size by a factor $\sim (v_0/c)$. In 'oriented' screen models, the screen is either localised along the line of sight or a large section of it is almost tangent to one of the retarded ovoids. In this case the observed size can be comparable with the actual size and the duty cycle increased to about unity, but only at the expense of requiring the observer to have a particular orientation with respect to the screen [with probability $\sim (c/v_0)^2$].

One can perform similar calculations for more complex geometries. The results obviously depend sensitively on the shape and orientation of the screen and somewhat on v_g . (Note that in the special cases of the sphere and the ring, the kinematics is independent of v_g .)

In Fig. 2 we display the results of a calculation of the observed shape when a spherical pulse moving with a speed $v_g \sim c$ illuminates an axisymmetric screen tailored to produce an expanding double. In this model, separation speeds $\lesssim 5c$ can be seen with most observer orientations.

The third type of causal explanation, which we term a 'phase' model, arises when v_ϕ and v_g are mutually parallel, and approximately perpendicular to the line of sight. This configuration occurs when, for instance, a synchrotron source is assumed to trail behind a moving object^{38,39}, expanding transversely to the direction of motion. The point of maximum brightness can move with $v_\phi \gg c$ even when $v_g \ll c$; and, in a suitable geometry, an expanding double source can be produced. However, in order that the regions of highest brightness separate with an observed velocity $v_0 \gg c$, it is necessary that the object move along a line lying almost in the plane of the sky, so that $\cos\theta_\pm \sim (c/v_0)(1 \pm v_\phi/v_0)$. This will occur with a probability $\frac{1}{2}(\cos\theta_+ - \cos\theta_-) \sim c/v_0$ (using equation (2)). For orientations outside this range, light travel time effects will reduce and may even reverse the observed velocity. Again this is a serious problem for this type of model as it either requires the sources to be in a super-luminal mode for a small fraction of their life, or that most expansions be non-relativistic. However, it does have an advantage over ballistic and screen models in that the actual source size can be comparable with what is observed ($\sim v_0 t$).

Acausal models

Changes in VLB structure need not, however, be attributed to a single agency. For instance, 'Christmas tree' models^{40,41} have been proposed in which independent sources (lying in a disk, viewed approximately equatorially) light up successively so as to

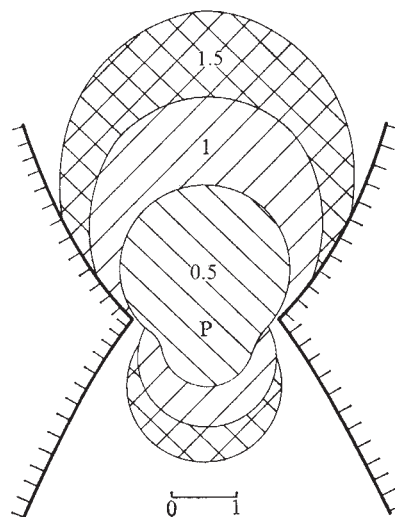


Fig. 2 Specific example of a 'screen' model. A central 'powerhouse' P, such as an accreting black hole or a compact cluster of pulsars, is presumed to be responsible for the production and maintenance of two anti-parallel beams of relativistic fluid. The walls of these beams are taken to be confocal paraboloids of unit semi latus-rectum. A shock with speed $\sim c$ travels radially outwards from P and radio emission is presumed to occur after this shock hits the channel walls for a duration of $0.5/c$. The observer is inclined to the symmetry axis with the most probable inclination of 60° . The emitting material is pushed radially outward with a mild relativistic speed ($\sim c/3$) so that the radio emission is bright only from those parts of the wall where the radius vector makes an angle $\lesssim 80^\circ$ (corresponding to $\mathcal{D} \sim 1$) to the observer direction. The diagram shows the shapes observed at times $t = 0.5/c, 1/c, 1.5/c$. VLB observations of this outburst with a linear resolution ~ 2 would probably be interpreted in terms of an expanding double source in which the separation speed decelerates from $\sim 5c$ to $\sim 4c$. The flux from the two sources would be comparable, the reduced area in the lower component being somewhat compensated by an increased surface brightness resulting from it being closer to P. Of course, the particular details of this model are *ad hoc*, but it does demonstrate that a super-luminally expanding double source can arise quite naturally in the twin beam geometry. Separation speeds $\sim 10c$ can be achieved if the orientation has a less probable value $\lesssim 30^\circ$, but in this case the observed brightness distribution will probably be less symmetrical and the variation with time more dramatic.

mimic a regular expansion. One objection frequently raised to these models is that only expansions, rather than equal mixtures of expansions and contractions, are generally observed. However, as an example, if there are three stationary radio sources aligned so that they are not quite resolved and the central component flares irregularly with a rise time much shorter than the decay time, whilst the outer components have the time-reversed behaviour, then apparent expansions will usually be observed³⁸. In not all sources is the resolution adequate to exclude this type of model.

Doppler effect

The foregoing discussion has been concerned primarily with the rate of expansion of the projected source dimensions and involved only the source geometry and the effective value of γ_g . The relative surface brightness of different parts of the source depends on various astrophysical effects—particle acceleration, magnetic field strength, energy and isotropy of the hypothetical central outburst and so on, of which our knowledge must be limited. However, one important effect which we can consider is the Doppler shift. Its parameters are given by the factor $\mathcal{D} = [\gamma_{em}(1 - (v_{em}/c) \cos\theta)]^{-1}$ where θ is the angle between the velocity v_{em} of the emitting material and OP. The intensity from a given element of proper

volume is then $\propto \mathcal{D}^{3+\alpha}$ (ref. 42) where α is the spectral index. When the material responsible for the emission shares the group velocity, that is, $\gamma_g \sim \gamma_{em}$, then $\mathcal{D}$ ranges from $\gamma_g(1+v_g/c)$ to $[\gamma_g(1+v_g/c)]^{-1}$ for motion parallel and anti-parallel to OP. On the other hand, if there is no bulk motion and v_g is, for example, the group velocity of a low frequency wave pulse, then there is no Doppler shift. We would argue that the true situation lies between these two extremes which are both difficult to reconcile with the observations.

For ballistic models in which $\gamma_g \sim \gamma_{em} \sim \gamma_0$, those parts of the source which display the largest superlight velocities would all have $\mathcal{D} \sim \gamma_{em}$ and this enhances the probability of observing the effect. Indeed one can even envisage a 'shrapnel' model¹⁰ in which $\sim \gamma_{em}^2$ sources are ejected in random directions but the two with $\theta \lesssim \gamma_{em}^{-1}$ are so strongly highlighted that only an expanding double (with, however, a randomly oriented axis) is seen. On the other hand, one cannot plausibly interpret a super-luminal expanding double as two plasmoids moving in opposite directions unless their proper emissivities decay coincidentally as $\tau^{-(3+\alpha)}$ (ref. 43) where τ is the proper time. (If this were the case then the total flux density should fade $\propto d^{-(3+\alpha)}$ where d is the component separation, a possibility that can already be excluded for 3C273.)

The hypothesis that $\gamma_g \sim \gamma_{em}$ then runs into difficulties because it would give rise to a very non-uniform surface brightness making it particularly difficult to account for equal doubles aligned along a preferred axis. However, if $v_{em} \ll c$ and the Doppler effect does not apply, as can occur in both screen and ballistic models, super-luminal expansion will last only a small fraction $\sim (c/v_0)^2$ of the light travel time across the emitting region. So if one averages over a time interval larger than the light travel time, then only $\sim (c/v_0)^2$ of the flux reaching the observer would be associated with an apparent speed v_0 . Moreover, this applies at every instant if, as seems to be the case for 3C120 (ref. 44), the outbursts seem to overlap. Present observations indicate that the flux from the high speed components is not dominated by a slowly changing background so that some bulk relativistic motion of the emitting material must occur. Note that even a velocity of $v_{em} \sim c/3$ would give brightness varying by a factor $2^{3+\alpha}$ as θ increases from 0 to π . In most sources, the source brightness temperatures directly measured by VLBI are $\lesssim 10^{12}$ K. This implies that only mildly relativistic¹² ($\gamma_{em} \lesssim 2$) bulk Lorentz factors are necessary to evade the self-absorption and inverse Compton constraints in incoherent synchrotron emission models. This is consistent with a model such as that illustrated in Fig. 2 in which the kinematics is dominated by the shape of the screen rather than by bulk motion.

In phase models displaying super-luminal expansion $\theta \sim \pi/2$ and the variation in $\mathcal{D}$ across the source is unlikely to be important even if $v_{em} \sim c$.

Propagation effects in extrinsic models

In extrinsic models, the rapid expansions are attributed to radio propagation effects: a small source switches on, the first radio emission to reach the observer follows a direct route and the apparent expansion results from radiation which is focused or scattered towards the observer after traversing increasingly curved paths with consequent progressively larger time delays. If the radiation is bent through a small angle ϕ while propagating out to a distance r , the apparent size would be $\sim 2\phi r$ and the expansion velocity $v_0 \sim 2c/\phi$. The objection to isotropically scattering stationary screens—that the flux be dominated by back scattered radiation—does not apply in this case as long as $\phi \ll 1$.

In 'local' models, processes operating on scales of tens of parsecs must scatter, reflect, or refract radio wave through angles of a few degrees. However, this can usually only be done at the expense of restricting the size of the central source

of radio waves, assumed to be roughly spherical, to a fraction $\sim c/2v_0$ of the observed size, thus increasing the brightness temperature and exacerbating the 'inverse Compton catastrophe'. By contrast in intrinsic source models the observed source size is typically a lower bound on the total linear dimension.

One specific mechanism that could operate in this way, and is probably at least a complicating factor in other models, is induced Compton scattering⁴⁵. If the brightness temperature of the central source is $T_b > m_e c^2/k \sim 10^{10}$ K, then a surrounding cloud of non-relativistic plasma would have an optical depth to induced scattering of $\tau_{ind} \sim (kT_b/m_e c^2)\phi^4 \tau_{spont}$ (ref. 46) where τ_{spont} is the optical depth to spontaneous Thompson scattering. If $\tau_{ind} \gtrsim 1$ while $\tau_{spont} \ll 1$, only induced scattering will be important and this is restricted to those directions that are already occupied by photons. So in such a model there would have to be a background source of angular size comparable with the maximum observed angular separation the brightness distribution from which would be supplemented by photons contributed by the rapidly fluctuating component. However, as radio brightness temperatures do not much exceed $\sim 10^{12}$ K, induced scattering cannot explain expansion speeds $v_0 \gtrsim 6c$ without invoking a specialised geometry. It also seems difficult to produce an equal double by this mechanism unless the observer happens to be located close to the equatorial plane. Nevertheless, induced scattering does involve a systematic lowering of a photon's frequency, and so a general softening of the radio spectrum is expected as the outburst develops⁴⁵, as is indeed frequently observed. (In extreme conditions, a small increase in radio brightness temperature can even occur).

In an alternative scattering mechanism, radio photons of wavelength λ can be deflected through an angle $\delta\phi \sim \lambda/\lambda_p$ by scattering a longitudinal plasmon of wavelength λ_p (ref. 47). If the turbulent energy density in the plasma surrounding the radio source can be maintained at a level sufficient to scatter each radio photon through $\sim 10^\circ$ then again an apparent expansion will be observed following an impulsive outburst. However, as the scattering cross section per electron $\propto \lambda^2$, the effect is strongly frequency-dependent. When the plasma turbulence is approximately isotropic, the maximum scattering angle $\phi \propto \lambda^2$; and when the plasmons are unidirectional, as they would be if they had a discrete origin separate from the radio photons, then $\phi \propto \lambda^3$. So on these models if most of the radio flux is resolved into an expanding source with speed v_0 then the effect should largely disappear if the frequency is reduced by factors $\sim (c/v_0)^{1/2}$, $\sim (c/v_0)^{1/3}$ respectively, as the observed emission will then be dominated by large angle scatterings from earlier outbursts. Provided that the plasmon phase velocities $\ll c$, the frequency shift associated with these scatterings will be negligible and so the spectral variation in an outburst would have to be intrinsic to the source.

A third possibility is the small angle refraction of radio waves by a surrounding cloud of dense ionised filaments, possibly associated with optical emission lines. (In order to produce an expanding double aligned with the symmetry axis, either the individual filaments must be elongated in the equatorial direction or the distribution of scatterers would have to be extended along the axis.) Scattering angles $\sim 10^\circ$ can be plausibly achieved at cm wavelengths using typical emission line region parameters, but again as the effect is $\propto \lambda^2$ any prominent angular structure should disappear on trebling the wavelength. The available evidence does not support this prediction, which would also include strong deceleration.

A somewhat different local effect might be the obscuration of part of a spherical source by an equatorial disk of free-free absorbing plasma. In this way a closely separate double can be produced. However, in order to see a widely separated equal double, the observer must lie in the equatorial plane.

A possible non-local effect is the operation of a gravitational lens^{48,49}. If a compact mass lies along the line of sight it yields a 'double crescent' image. An angular scale of milli-arc seconds

requires a lens mass $\sim 10^5 M_\odot$. A lens model has the virtue that the components are generally of comparable flux density whenever the magnification is high. However, it does not seem possible to achieve systematic expansion by more than the unmagnified source size, and there would be no reason for expecting compact doubles produced in this manner to display any preferential alignment with the axis of an associated double source. An alternative non-local effect involves intergalactic scintillation associated with fluctuations in the density of plasma in the intergalactic medium or an intervening galaxy⁵⁰. This effect would operate similarly to local scintillation and again would be expected to be strongly frequency dependent.

Observational tests

We have analysed the most important features of several different models designed to explain the prime observational fact of extragalactic VLBI—the prevalence of compact double radio sources expanding super-luminally along a preferred direction. In fact, the observational evidence for this property is derived as much for composite studies of several sources as from detailed investigations of individual nuclei and so must still be treated with considerable caution. Nevertheless, improvements in techniques and processing capacity within the next few years should clarify the picture. On the basis of this feature, we have argued that the most attractive theoretical explanations (that is, those least incompatible with existing observations), involve the illumination of an appropriately shaped screen (for example, the cylindrical walls of two anti-parallel relativistic beams) by a 'signal' moving at the speed of light (for example, a relativistic blast wave or a pulse of low frequency radiation), originating from a more compact central source (which might be a $10^6 M_\odot$ black hole). In these models it seems necessary that the emitting region move outwards with a mildly relativistic speed. The arguments advanced against alternative 'intrinsic' models are mainly probabilistic; and most 'extrinsic' models be ruled out because they predict an apparently unobserved frequency dependence. These arguments too must be treated with scepticism at this stage. We conclude by listing some additional observational tests and outlining the conclusions that it may be possible to draw from them.

(1) Do systematic source motions always correspond to expansions rather than contractions? If so it would form the strongest evidence against naive acausal 'Christmas tree' models. It would be of particular interest to know to what extent successive expansions overlap in time and what fraction of the total compact component flux is associated with moving components, as this would indicate how rapidly the emitting region would have to recover so as to accommodate the next outburst, and how rapidly it would have to expand so that only the front of the source would be observed.

(2) Does the position angle and/or the expansion speed vary during a single outburst? Models involving relativistic bulk motions can produce either accelerating or decelerating expansions. Indeed for those sources where the component separation is observed to expand by a factor ~ 2 , it would be surprising if some change in the observed velocity were not seen. Blast wave models are generally decelerating, whereas free expansions, which are perhaps unlikely in view of the rapid decline with time of their optically thin emission, may accelerate. The expansion rates for screen models are determined mainly by the shape and orientation of the screen, but if the outburst or screen are not axisymmetric, the position angle may change³³. One interesting possibility is that the velocity be accurately constant during an outburst. In this case the expansion would probably have to be attributed to one or more coherent objects moving inertially, although in certain conditions blast wave models can exhibit constant velocity phases²¹.

(3) Are the position angle and expansion speed 'remembered' from one outburst to the next? Successive outbursts should

display the same alignment if they represent expansion along a preferred direction (for example, the rotation axis of a central massive object or rotating gas cloud). A reproducible expansion speed would be a strong argument in favour of a screen model, but the absence of such would not necessarily rule it out because the signal velocity v_s could vary between outbursts, which would alter the kinematics.

(4) What are the absolute positions of the components and which ones are moving fastest? Clearly the development of phase stable VLBI would require a considerable technical advance. However, it may be possible to learn which parts of the source are moving if either the nucleus can be resolved into two or more components, one of which is unchanging, or, in the case of triples, if it is assumed that the central component is stationary. Alternatively, for those sources for which there is a sufficiently nearby unresolved radio source (for example, 3C345/NRA0512), it may be possible to obtain some absolute positional information.

(5) How do the components of expanding doubles evolve? If the emission is due to synchrotron radiation, the component size must always be large enough to bring the brightness temperature below the plausible upper limit set by the inverse Compton restriction. It would be important to know how the source size or surface brightness distribution depends on wavelength, and also whether or not a particular component faded out earlier and was a greater fraction of the flux at short as opposed to long wavelengths, as this would be a particular signature of propagation effects. Other models make specific predictions about wavelength dependence³⁹. Some models also make rough predictions about the distribution of surface brightness^{30,51}. For instance, in the twin-beam model^{18,52} illustrated in the figure, we might expect source asymmetry to increase with the apparent velocity. In simple screen models, the faster component would have lower brightness, whereas in models where the Doppler effect is more important, the reverse would be true. In sources showing successive outbursts, higher sensitivity observations would allow an old and fading component to be followed in the presence of a young and bright source. This could considerably improve the interpretation of the kinematics.

(6) What are the polarisation properties of the individual components? If as proposed on some models the emitting electrons are channelled along an effectively rigid magnetic field, then the observed radiation should be strongly polarised normal to the source axis if it is synchrotron radiation³⁶, and parallel if it is curvature radiation³⁷. If the field direction makes a small angle to the line of sight then perhaps a substantial circular polarisation⁵³ could be observed (unless electrons and positrons are created in equal quantities). The induced Compton and plasmon scattering models also predict polarisation directions related to the source geometry.

(7) Can we identify the time when a given outburst was initiated? Brightness temperature limitations generally preclude the possibility of seeing the start of an outburst at radio wavelengths. However, an unusual change in the optical or X-ray output may serve to identify the time origin (for example, in 3C273) which is particularly important in the case of screen models as this would allow a unique inversion of the geometry of the screen if the signal velocity is taken to be c .

(8) Is the position angle of compact double structure aligned with extended radio structure? So far the evidence for this is positive and so this constitutes an argument in favour of the extended sources' being fuelled continuously⁵⁴ by their central components. In these cases it is tempting to suppose that both sources share a common axis defined by the angular momentum of the central regions. However, as yet amongst these sources, only in 3C273 is there evidence for super-luminal expansion and it is very important to discover more examples. This is because, in most extended double radio sources, one can be fairly sure that the axis does not make a small angle with line of sight. So if rapid expansion, with $v_0 \gg c$ are found to be fairly common, it would probably rule out both ballistic and simple

screen models. One important source to study in this context is Cygnus A because in this case there is fairly convincing evidence that the source axis lies in the plane of the sky⁵⁵.

(9) What are the statistical properties of a sample of compact sources? It is already known that $\geq 10\%$ of flat spectrum 3C sources display super-luminal expansion. In some models where ejections occur in a particular direction and Doppler effects are important, the compact components would appear undetectably faint unless our line of sight lies in a privileged range of solid angle. As we have discussed, the probability of this may be related to the observed expansion speed in which case it should be possible to rule out this type of model. (Note, however, that there is a possible selection effect in that sources exhibiting large Doppler shifts will be preferentially included in a catalogue such as 3C.) We doubt that any models of the screen or ejection type could accommodate regular expansions with speeds $v_0 \gg 10c$ in more than a very small fraction of a large sample of sources. If these velocities were observed then they should motivate attempts to develop less unsatisfactory forms of models in alternative categories. On a more ambitious level, if sufficient understanding of the kinematics of a class of sources can be achieved, it may be possible to invert the problem and set limits on plausible values of the Hubble constant (or conceivably q_0).

Finally, we would stress the importance of carrying out systematic multi-frequency source monitoring using a standard (although necessarily somewhat arbitrary) algorithm for converting raw visibility data to source models, and of pursuing programmes that use the same combination of telescopes for successive observations.

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articles

Structure of RNA and RNA binding site in tobacco mosaic virus from 4-Å map calculated from X-ray fibre diagrams

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The structure of tobacco mosaic virus has been solved to a resolution of 4 Å using fibre diffraction methods. The general fold of the protein is clear, the conformation may be deduced in many places, and the conformation of the RNA and its interaction with the protein can be seen.

TOBACCO mosaic virus (TMV) is a rod-like virus, 3,000 Å long and 90 Å in radius, with a central hole of radius 20 Å. The identical protein subunits (molecular weight 17,500) build

a helix of 49 subunits in 3 turns, protecting the RNA. The particles form very well oriented gels^{1,2} which give X-ray fibre diffraction patterns of unsurpassed quality^{2,3}. The first successful calculation of a three-dimensional electron density map was by Barrett *et al.*⁴; however, they limited their analysis to the cylindrically symmetric part of the diffraction pattern, with a resolution of 10–15 Å. They pointed out that with sufficient heavy atom derivatives, the diffraction pattern could in principle be analysed at higher resolution, and Stubbs and Diamond⁵ showed that this could indeed be done. Holmes *et al.*³ applied these methods, to produce a map at 6.7 Å resolution. We report here an extension of the 6.7-Å work: data have been collected to 4 Å resolution and the heavy atom positions have been further refined, producing significant improvements in

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Table 1 Heavy atom derivatives

Ligand	Strain	Site no.	Residue	<i>f</i>	<i>r</i> (Å)	φ (deg)	<i>Z</i> (Å)	<i>R</i> (%)
OsO ₄	<i>Vulgare</i>	1	1	80	92.0	5.5	8.5	12.7
Pb ²⁺	<i>Vulgare</i>	1		50	26.0	6.5	20.2	12.3
UO ₂ F ₆	<i>Vulgare</i>	1	27	70	57.2	15.5	12.6	15.0
		2		50	26.8	5.9	19.1	
		3		55	57.5	22.3	25.4	
SHIMS-MMN	<i>Vulgare</i>	1	68	60	72.4	5.7	— 1.5	16.6
		2	27	70	56.7	15.5	12.6	
MMN	<i>Vulgare</i>	1	27	70	56.9	15.5	12.6	15.0
MMN	Ni-2068	1	27	70	57.1	15.5	12.6	13.8
		2	139	70	72.6	0.4	0.5	

SHIMS, sulphydryl imidoester³²; MMN, methylmercury nitrate⁸. Units of *f* are arbitrary, and correspond approximately to electrons. *R* is a least-squares *R* factor³³. *r*, φ and *Z* are cylindrical polar coordinates.

the electron density map. The quality of the inner part of the map seems to be what would be expected from a good 4 Å map and this has encouraged us to fit molecular models for the protein and the RNA to the map. We are considerably helped by the fact that most of the RNA binding site is composed of α-helix. We find little ambiguity in the way the atomic models can be fitted to the electron density map. For technical reasons the resolution of the map falls beyond about 60 Å radius so that we have not attempted to build detailed models for this region, although the trace of the polypeptide chain may be seen for the whole of its length.

The application of heavy atom isomorphous replacement to the fibre diagram of TMV has been described^{3,5}. The essential steps are data collection^{3,6}, location of heavy atom sites, calculation of phases and separation of Bessel functions, and Fourier-Bessel inversion⁸. These last two steps are equivalent to reconstructing three-dimensional diffraction data from the observed two-dimensional data. The nature of cylindrically averaged data precludes the use of Patterson methods in locating heavy atoms. Consequently, heavy atom location has been one of the major obstacles to progress in this work. The methods finally used depended on determining the occupancy and radius of a heavy atom from the zero layer line⁷, then finding the other coordinates from the remaining layer lines^{3,8}. Single site derivatives were essential in the early stages, as were double derivatives of these. The heavy atom derivatives used are described in Table 1.

The phase problem for cylindrically averaged data differs from that in crystallography. The intensity of diffraction is⁹⁻¹¹

$$I = \sum_n G_n G_n^* \quad (1)$$

G is a vector analogous to the crystallographic *F*. The allowed values of *n* depend upon the helical symmetry of the particle. Three terms of equation (1) should be considered for TMV at 4 Å resolution. Thus the intensity contains six unknown quantities, the real and imaginary parts of three *G_n*, c.f. whereas in crystallography there are only two unknowns. The solution of this multi-dimensional phase problem has been described by Stubbs and Diamond⁶ and Holmes *et al.*³

Electron density map

The map shows clearly recognisable right-handed α helices and resolved sugar and phosphate peaks in the RNA. We have examined 4-Å maps of proteins and a 4-Å map of tRNA produced by Rich and his colleagues, and conclude that by current standards, this is a good protein map, and a particularly good RNA map for this resolution. The exception is the region at radius greater than 65 Å, where the resolution is reduced in the azimuthal direction, falling to 5.5 Å at 80 Å radius (G. S. & S. W., unpublished). However, parallel studies on the crystallised disks of the coat protein¹² will yield detailed information about the outer region, so we have not attempted a detailed fit beyond 60 Å radius.

The RNA stands out as the strongest and clearest feature.

It follows the virus helix at a radius of about 40 Å, as has long been known³⁴, but it is considerably puckered, and in fact lies between 35 and 50 Å. It is a continuous chain, but with a number of interactions with the protein density, and three protruding features which we identify as bases. There are clearly separated peaks along the chain, interpreted as sugar rings and phosphates. The RNA density (without two of the protrusions, which are obscured by the main chain in this view) is shown in Fig. 1a.

The protein chain (158 amino acids) can be traced with confidence for about 70% of its length, from residue 20 to 55, and from 69 to 140. The connecting peptide, the N-terminal chain, and the C-terminal chain are present, but details are less certain, since these all lie at high radius. There are four α helices running approximately radially, which have been described at lower resolution³ and have also been seen at lower resolution in the crystallised coat protein¹². We adopt the descriptive terminology of Champness *et al.*¹² and call these the left and right radial helices (LR and RR), and the left and right slewed helices (LS and RS). Part of the electron density corresponding to the left radial helix is shown in Fig. 1b. It is clearly a right-handed α helix (confirming our choice of hand, made on the basis of the electron microscopy results of Finch¹³) and most of the side chains are marked by density, although not, of course, sufficiently well to recognise them. The same is true of the left and right slewed helices. The right radial helix is not as clear as the other three but is recognisable by its long, straight form, its size and density, and a certain amount of periodicity.

There is also a short stretch of helix at 23 Å radius, the innermost boundary of the protein. We call this the vertical helix (V). This runs parallel with the particle axis, and packs closely with its equivalent helices in adjoining subunits to line the central hole of the virus. Figure 1c shows the density at low radius, where three helices including this one form a bend.

Although the connectivity of these helices was not correctly ascertained at 6.7 Å resolution in the virus³, it was clear at 5 Å in the protein crystals¹² and the chain tracing of Champness *et al.*¹² is confirmed here at 4 Å. In addition, we can trace the chain at low radius, a region which lacks density in the protein map, probably because of disorder¹². The LS helix, beginning near Pro 20, runs inward. It may be slightly shorter than its counterpart in the absence of RNA (see Fig. 1 of Champness *et al.*¹²). It connects to the second helix (RS), which runs from about 50 Å radius outwards, then the chain goes through RR to very low radius, almost meeting the vertical helix, after which the LR helix leads to high radius at about residue 135. The remaining residues are at high radius.

Model building

Protein model building at 4-Å resolution is not normally possible. In the case of TMV, however, many constraints are provided by the abundance of chemical information. Moreover, the map is very clear. The locations of residues Ser 1, Cys 27, Lys 68, and Tyr 139 were fixed by the binding of specific heavy

atom derivatives at those sites^{4,7}. The C terminus is known to be on the outside surface because it is accessible to carboxypeptidase¹⁴. Especially useful were the sequences of over 100 mutants and strains of TMV, indicating which residues are invariant. It was helpful to know the 5 Å structure of the crystalline protein disk¹², which clearly shows the path of the

polypeptide chain connecting the LS and RS helices (residues 31–41), a region which is ambiguous in our map. A most important factor in our ability to build a model was the fact that at radius < 60 Å, TMV is mostly α helical. The atomic model was built with Nicholson push-fit models (1 cm to 1 Å) in a Richards box¹⁵.

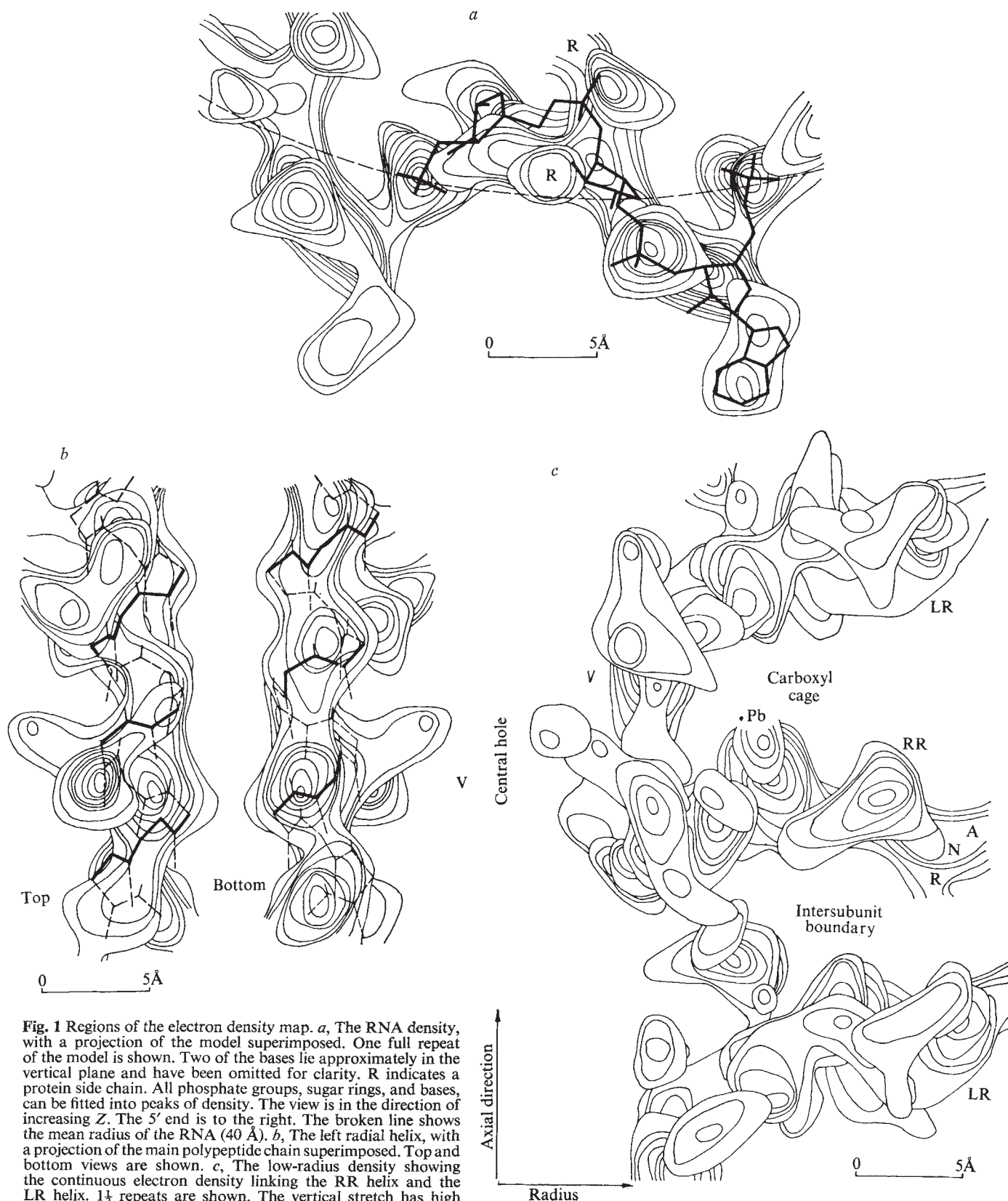


Fig. 1 Regions of the electron density map. *a*, The RNA density, with a projection of the model superimposed. One full repeat of the model is shown. Two of the bases lie approximately in the vertical plane and have been omitted for clarity. R indicates a protein side chain. All phosphate groups, sugar rings, and bases, can be fitted into peaks of density. The view is in the direction of increasing *Z*. The 5' end is to the right. The broken line shows the mean radius of the RNA (40 Å). *b*, The left radial helix, with a projection of the main polypeptide chain superimposed. Top and bottom views are shown. *c*, The low-radius density showing the continuous electron density linking the RR helix and the LR helix. $1\frac{1}{2}$ repeats are shown. The vertical stretch has high density and may be fitted to a 10-residue α helix (V) which is terminated by Pro 102 at the N-terminal (RR) end,

Table 2 RNA conformation—angles used for model building

Nucleotide	ϕ (deg)	ψ (deg)	θ (deg)	ξ (deg)	σ (deg)	ω (deg)	χ (deg)
1	70(g^+)	145(t)	220(t)	170(t)	135(C2'-endo)	250(g^-)	110(<i>anti</i>)
2	275(g^-)	40(g^+)	185(t)	195(t)	135(C2'-endo)	210(t)	170(<i>anti</i>)
3	290(g^-)	150(t)	135(t)	270(g^-)	135(C2'-endo)	210(t)	105(<i>anti</i>)

The angle values are unrefined and thus somewhat uncertain. For definitions of angles see Arnott (ref. 16).

The RNA

The RNA has three nucleotides per protein subunit. In the map there is a separate local maximum for every phosphate, every ribose and every base. In order to be certain that only one RNA structure would fit the map, we systematically tried and rejected all but one of the permutations of previously observed conformations of the variable angles. Following the notation of Arnott¹⁶, we used the constraints (1) (ϕ , ψ) $\simeq$ (180°, 180°) is not allowed^{17, 18}. (2) $\xi \simeq 300^\circ$ implies $\sigma \simeq 140^\circ$ (C2'-endo)¹⁹. (3) All bases are *anti*^{16, 18}. (4) All other angles are within or near the previously observed ranges for these angles, as compiled by Arnott¹⁶. (5) Only C2'-endo and C3'-endo conformations were tried as the pucker of the ribose ring; the other possible puckers were judged to differ so slightly from one or the other of these as to be indistinguishable by our methods. (6) Both possible directions of the RNA chain were pursued.

Each conformation tried was set first at a nominal angle and then small variations of the angles were used to improve the fit if possible. Of the approximately 130,000 possible permutations, only about 1,000 had to be tried because many trials were rejected after setting the first few bonds. We were only able to find one conformation which fitted the density. As a check we note that this conformation has the property of being able to repeat as a continuous chain into the next subunit, as required. The geometry of the RNA is given in Table 2. A projection of the RNA, superimposed on its electron density, is shown in Fig. 1a. All three sugars were found to have the C2'-endo conformation. This conformation is found in B-form double helical DNA¹⁶ (ref. 16) and in non-helical regions of tRNA (ref. 18). The three phosphates are at radii 37, 39.5, and 42 Å. The 5' end is at the concave end of the particle. This result has been independently obtained from electron microscopy by Wilson *et al.*²⁰.

The protein

Each helix was first fitted as a polyalanine helix to establish the course of the backbone and to fit β -carbons into bumps of density.

Left slewed helix, residues 21–31—Pro 20 was placed at the bend where the helix starts. The phase of the helix was specified by requiring Cys 27 to be near the MMN site.

Right slewed helix, residues 40–53—Pro 54 was placed at the bend ending this helix. The phase of the helix was chosen to place Phe 48 and Trp 52 into density peaks corresponding to their shapes. This placed Arg 41 near phosphate 1, Glu 50 on the intersubunit boundary, and a hydrophobic face toward Cys 27.

Left radial helix, residues 114–134—The coordinates of residue 139 were known from a specific heavy atom derivative at that site⁴. This site is 22 Å from the corner which ends the LR helix, so the corner residue is likely to be in the range 126–132. A side chain bump of density near that corner, one turn in from the high radius end of the helix, seems to be a ligand for the third UO_2F_5 site. Residues 128–134 are all hydrophobic except for Glu 131 and Arg 134. Since carboxylic acids are known to bind UO_2^{2+} , we placed Glu 131 at that site, which then specified the phase of the LR helix. This positioning required the external face of the helix to be surprisingly hydrophobic. We see below, however, that it functions as a hydrophobic binding site for the RNA bases.

Vertical helix, residues 103–112—It was already known from the radial density distribution of TMV reconstituted protein^{21, 24}

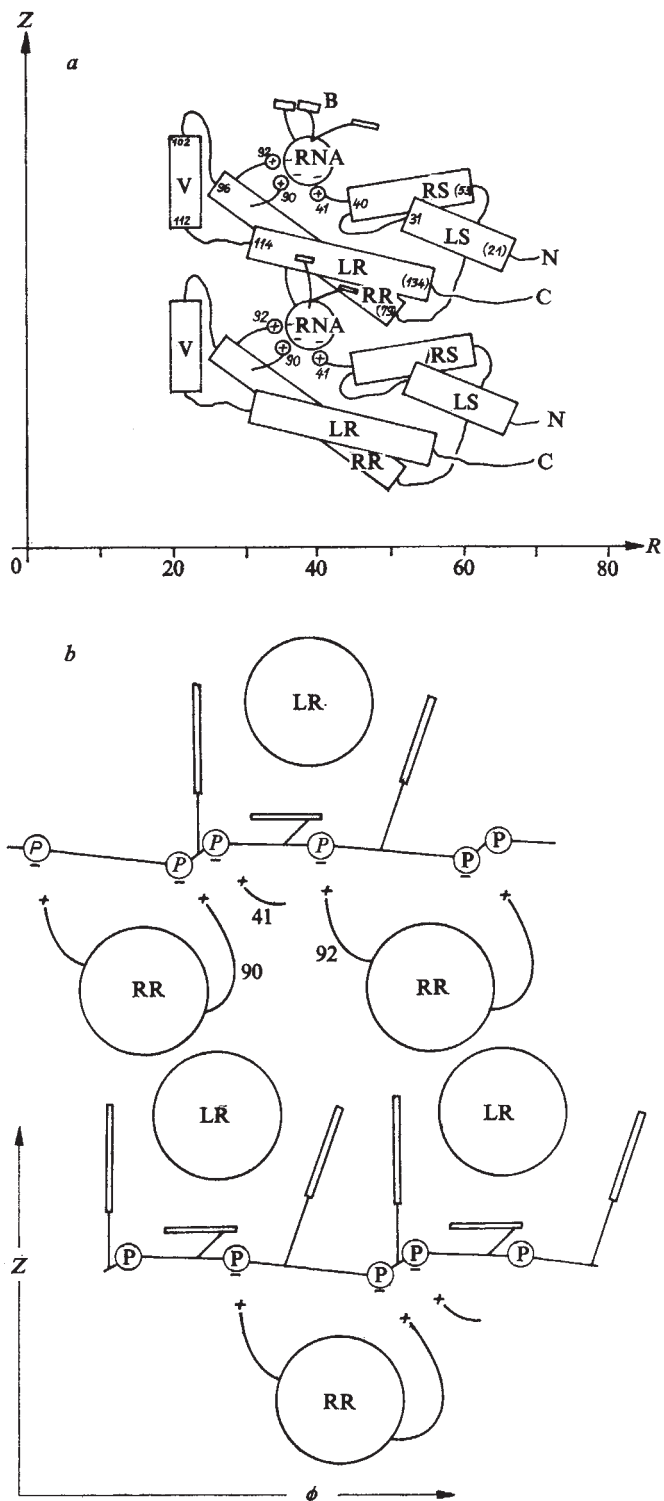


Fig. 2 *a*, Schematic drawing of the inner part of TMV. The carboxyl cage is formed by the left radial, the right radial, and the vertical helices at a radius of about 30 Å. The view is in the direction of increasing ϕ . B indicates an RNA base. *b*, The RNA binding site viewed along a radius. The residue numbers of arginines which seem to be forming salt bridges to the phosphates are given. Note how the three bases form a claw which grips the hydrophobic LR helix: LR, left radial helix; RR, right radial helix; P, phosphate.

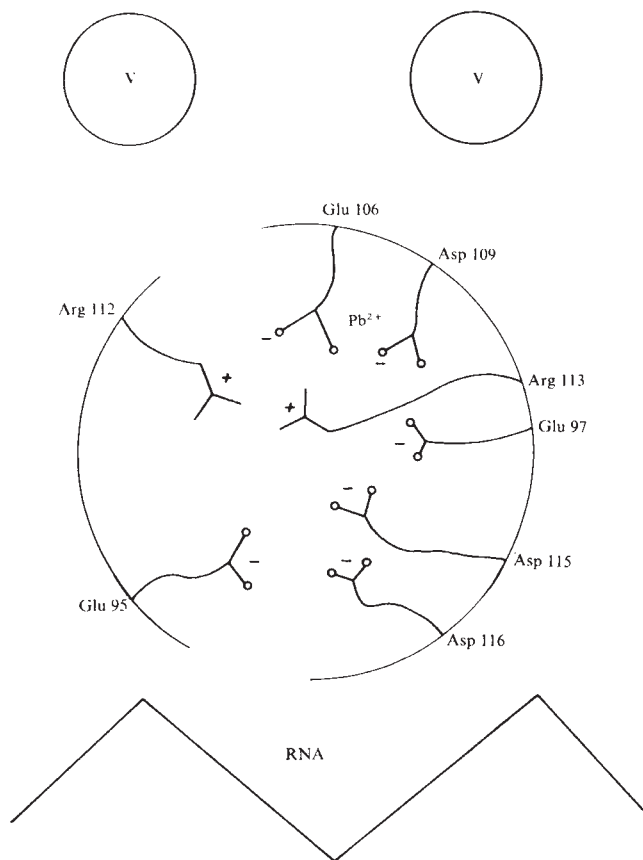


Fig. 3 Schematic drawing of the carboxyl cage as seen from above.

that the region at 23 Å radius was the densest part of TMV protein. Here we see density which we attribute to a 10-residue α -helix parallel to the cylinder axis. At this radius the 22° wedge-shaped subunit is only 9 Å wide, just the proper spacing for close packing of the vertical helices from adjacent subunits. The important constraint on this helix was that Pro 102 should not be built into it. To connect V with LR (and later to pack residues in density between V and RR), we put Pro 102 at the beginning of the V helix. This choice is supported by the fact that this helix is absent or greatly reduced in size in the U2 strain of TMV²² (and S. Morris, G. S. & S. W., unpublished) which has a proline as residue 107. The phase of the helix was specified by fitting the proper side chains best into density. This fit resulted in the Pb binding site being located between carboxyls Glu 106 and Asp 109 and placed four threonines (103, 104, 107, 111) on the surface exposed to solvent of the central hole (radius 19 Å). Since this assignment of phase has important implications for the existence of a 'carboxyl cage' (see below) experiments with specifically labelled mutants are being undertaken to check this point.

Right radial helix, residues 79–96—The requirement that RR connect with V approximately specified the end residue of RR. The respective consequences of building RR to terminate at each residue in the range 92–101 were noted. The phase which provided the best knobs-into-holes packing with LR put the end of RR at 96. This phase also placed the charged head groups of Arg 90 and Arg 92 into density peaks near phosphates 2 and 3 respectively. At Pro 78, at the high radius end of the RR helix, the chain seems to make a slight bend (becoming steeper in Z) and then to continue out in radius as far as residue 70.

Connecting links—Plausible connecting links were built from LS to RS, from RR to V and from V to LR. For residues 98–101, (ϕ , ψ) pairs were set to be in any of the three major allowed regions of the Ramachandran plot²³ and all 81 permutations were tried. Only one was found which both fit the

density and spanned the required distance. The connection between helices LS and RS (residues 32–40) contains many polar side chains which probably form part of the intersubunit boundary toward the left.

Principal features of the structure

From the model it is evident that the part of the molecule we have built includes four significant regions: the packed helices at medium radius, the RNA binding site, a 'carboxyl cage' and the vertical helix. They are shown in Fig. 2a. We discuss them in order.

The packed helices—As has already been observed¹², the four approximately radial helices pack well together, making angles of 10–20° with each other. Our model has good knobs-into-holes interactions. In the vicinity of Cys 27, the helices form the walls of a hydrophobic pocket: the sulphhydryl is in the centre. The unusual unreactivity of the sulphhydryl has long been known²⁴, and is evidently caused by this environment. This cysteine is conserved in most strains and mutants although its function is not clear. In the cow-pea strain, however, it is replaced by a leucine,³⁵ and since this is of similar size, we might guess that the constraint on residue 27 is the packing of the helices.

The RNA binding site—The RNA lies between subunits in successive turns of the virus helix, so that the binding site is in two halves, between the top of one subunit and the bottom of the next (Fig. 2). One half is the base binding site, located almost entirely on the LR helix. This helix has a large number of hydrophobic aliphatic residues between 117 and 128, which form three faces for the bases. All can accommodate any type of base, since the bases lie flat against hydrophobic side chains. Several residues near the faces, including Thr 89, might form hydrogen bonds with the bases, but the model is not sufficiently accurate to make any definite statement. The other half of the site is mostly on the RR helix, and its principal function is to bind phosphate groups by forming ion pairs, using arginines 90, 92 and 41. The hydrophobic chain of Arg 41 might well be stabilised by interaction with Phe 87, which could occupy a peak of density between the RS helix and the RNA. We note that two other groups on the RR helix, Asn 91 and Asn 98, are in positions where they could form hydrogen bonds with phosphate oxygens, but at this resolution we cannot prove that they do so.

The carboxyl cage—Between the RNA and the vertical helix is the feature illustrated in Fig. 3. Six carboxyl groups are in a pocket which is otherwise lined with hydrophobic residues. The pocket is formed by the LR, RR and V helices of one subunit, and the RR helix of the neighbouring subunit. They are partly neutralised by Arg 112 and Arg 113 (coming from different subunits). It seems likely that lead and uranyl fluoride bind to two or more of these carboxyl groups, and that the carboxyls could form a binding site for other divalent cations. Such binding sites have been observed in other structures^{25–27}, but this is a somewhat unusual example, because of the number of carboxyls involved and because the carboxyls are apparently not fully neutralised by bound metal ions or basic side chains. (Nearby, but apparently not interacting with the carboxyl cage, are Asp 88 and Arg 122, which form an inter-subunit salt bridge.) Caspar²⁸ suggested the presence of pairs of carboxyl groups in TMV with anomalous pK values near 7, and this work was confirmed by Butler, Durham and Klug²⁹. There seems little doubt that the anomalous pK values originate in the carboxyl cage, where carboxyl groups are forced together in a hydrophobic environment, although the situation is apparently even more complex than simple pairs of carboxylic acids. The short vertical helices form a wall, part of the rigid carboxyl cage structure, and protect the RNA from the solution.

TMV assembly

From the above observations, we draw some inferences about the assembly and dissociation of TMV. Caspar²⁸ has suggested

that the carboxyls with anomalous pK values provide a sensitive switch, active in physiological conditions, between the dissociated and intact virus states. Considering the RNA binding region and the carboxyl cage together, we can speculate about the mechanism of this switch. The coat protein has two important states, helix-forming and disk-forming, which are delicately balanced in normal conditions. Switching between the states is controlled by pH , presence or absence of RNA, and possibly by concentration of one or more cations.

The mechanism of the switch seems to involve electrostatic interactions among six carboxyls, five arginines, and when present, three RNA-phosphate groups. The helical state exists either in the intact virus or at low pH . It is characterised by the presence of the interlocking V-helices which force carboxyls together in the 'carboxyl cage', accounting for the abnormal proton binding. In the virus, RNA interactions stabilise this state over a wide range of conditions, even above pH 7 where ionisation of some of the abnormal carboxyl groups can be induced. The disk may be obtained at neutral or high pH in the absence of RNA. Comparing our results with those of Champness *et al.*¹², the disk is characterised by having no V helix and only half the RR helix. Because of the collapse of the RR helix Arg 90 and 92 are presumably able to neutralise carboxyl groups. This rearrangement (involving about 30 residues) is seen as disordering at small radii in the disk-crystals. Through this mechanism the binding energy of the RNA is partially balanced against the repulsive energy of the 'abnormal carboxyls' which aids uncoating. In the low pH helical-aggregate of the coat problem, where protons establish electroneutrality in the carboxyl cage, the protein can adopt the helix structure, even in the absence of RNA (21, 34, and a 4-Å difference Fourier—E. Mandelkow, S.W. & G.S., in preparation). This suggests the formation of the V-helix may be the factor controlling the disk-helix transition.

Finally, in view of the solid wall formed by the interlocking V-helices, the disordering of the V-helix in the disk, which is an important precursor in the assembling of the virus, is probably necessary to enable the RNA to enter its binding site from the inside hole of the growing virus^{30,31}.

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Note added in proof: Further examination of our model shows that Arg 113 (from the subunit at higher Z) could interact with a phosphate of the RNA. This would take Arg 113 out of the carboxyl cage, and take Arg 41 away from the RNA. Arg 90 would bind to the high-radius phosphate, and Arg 113 to the low-radius phosphate. The structure would be otherwise unaltered. At present we are unable to distinguish between these alternatives.

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Transmembrane channel formation in rhodopsin-containing bilayer membranes

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Rhodopsin has been incorporated into planar lipid bilayer membranes. The effect of light is to increase the bilayer permeability in a pattern consistent with the formation of a transmembrane channel of about 10 Å diameter. A model of visual excitation based on a light-activated and voltage-sensitive channel is presented.

PHOTORECEPTOR cells in both vertebrate and invertebrate species are capable of converting a single absorbed light quantum into a neural signal^{1–3}. The photon is absorbed by rhodopsin, a chromophore-bearing membrane protein^{3,4}. The central problem, therefore, is to determine how a

detected photon can initiate an amplified excitatory response of the receptor cell. It is generally accepted that the light-induced changes in rhodopsin lead to cellular excitation by modulating the ionic conductance of photoreceptor cell plasma membranes^{2,3,5–7}. Such changes could result from a simple mechanism based on rhodopsin functioning as a light-activated ion translocator⁸. A direct way to test this possibility would be to incorporate rhodopsin into a membrane system amenable to the experimental methods required to investigate the postulated transport properties^{9,10}.

Here we describe the properties of planar lipid bilayer membranes with rhodopsin included, and show that light increases the membrane conductance in a pattern consistent with the formation of a transmembrane channel^{10–14}. We propose a heuristic model of visual phototransduction which accommodates the data so far obtained in photoreceptor cells.

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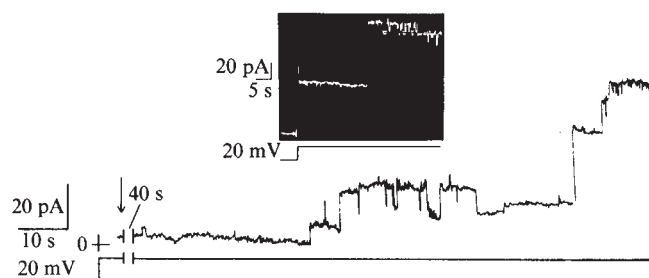


Fig. 1 Time course of the current transition from dark to bleached states. Planar bilayers were formed in a circular aperture of 0.26 mm in diameter as described before²⁹. The Teflon cell consisted of two compartments, each with an area of 3.5 cm². Calomel or Ag-AgCl electrodes were connected to a feedback current amplifier of the type previously described³⁰. The cell and the current amplifier were enclosed inside a light-tight aluminium box. A polarising d.c. voltage, of various amplitudes, was applied across the membrane, the current was displayed on a storage oscilloscope and simultaneously recorded on a strip chart recorder. Bleaching was accomplished with 500 nm light of approximately 10^{16} quanta cm⁻² s⁻¹. All bilayer experiments were done at room temperature. Rod outer segments from dark-adapted bovine retinas (Hormel) were isolated by sucrose flotation and purified in a discontinuous sucrose gradient³¹. Cetyltrimethylammonium bromide (CTAB) was used to extract rhodopsin³². All the steps were performed at 4 °C under dim red light unless otherwise stated. The rhodopsin proteolipid was prepared as follows: detergent (CTAB) solubilised rhodopsin (0.75 mg) was incubated with humid Bio Beads SM-2 (ref. 33) (90 mg) for 5–7 min at 24 °C, and then mixed with a dispersion of partially purified soybean phospholipids³⁴ 10 mg ml⁻¹ in 1.0 ml of 0.1 M KCl, 0.01 M imidazole buffer, pH 7.0. The mixture was sonicated by immersion of the test tube in a water bath sonicator³⁵ for 4 min at 4 °C. Then 0.1 ml of 100 mM CaCl₂ and 1 ml of hexane were added to the suspension. The tube was vigorously mixed for 4 min and the two phases were separated in a clinical centrifuge for 1 min. The hexane phase was removed and a second extraction was performed with 1 ml of ethylether; the suspension was mixed for 3 min and the two phases were separated. The two extractions were performed at 4 °C. The hexane extract exhibits a mean absorbance difference, ΔA_{500} (dark-bleached) of $\Delta A_{500} = 0.022 \pm 0.021$ ($n = 20$), whereas the ether extract presents a mean $\Delta A_{500} = 0.183 \pm 0.043$ ($n = 20$). An ether rhodopsin proteolipid with $\Delta A_{500} = 0.16$ was spread over 0.2 M KCl, 1 mM CaCl₂, 0.01 M imidazole pH 7.0. After evaporation of the ether the bilayer was formed. In the figure the membrane current at a voltage of 20 mV is displayed. The arrow represents the point at which the light was switched on.

Planar bilayers which separate two well defined aqueous phases offer the possibility of directly measuring electrical parameters: the time course of light-induced conductance changes together with direct evidence for channel existence can provide insight into the mechanism of rhodopsin action.

Rhodopsin-containing bilayers were made from the rhodopsin proteolipid in organic solvent as described in the legend of Fig. 1. Successful regeneration of rhodopsin treated by this procedure (yield ~70%) showed that it displayed normal spectral characteristics of the native form. The principle of hydrophobic apposition of two monolayers was used for bilayer formation^{9,10,13}. The absolute membrane conductance (G_m) in the dark was in the range $0.2\text{--}2 \times 10^{-9} \Omega^{-1}$; on illumination it increased to a variable extent. Figure 1 is a representative strip-chart recording of the kinetics of conductance transition from the level in the dark ($G_m = 2.1 \times 10^{-10} \Omega^{-1}$) to that in the light. The lag time between illumination and onset of the conductance transition is about 100 s. Estimated half-time of bleaching with the 500 nm light used here is about 3 s. Note that the conductance change proceeds in steps of magnitude of about $4\text{--}8 \times 10^{-10} \Omega^{-1}$ and also in bursts of large magnitude. The difference between the conductance steps and the bursts is more clearly illustrated in the inset, where an oscillographic recording of the same membrane is taken 330 s after illumination: the initial G_m is $32 \times 10^{-10} \Omega^{-1}$, the burst of con-

ductance of $40 \times 10^{-10} \Omega^{-1}$ and the fluctuations in the upper level of G_m are of $5.8 \times 10^{-10} \Omega^{-1}$.

The G_m transition from dark to bleached level may proceed in a 'noisy' way where no identifiable fluctuations can be resolved; this pattern was prevalent in the bilayers prepared from proteolipids with the highest rhodopsin concentration. G_m transition frequently proceeds in steps and bursts concurrent with a drift towards high G_m values. When the dark G_m was initially considerable, or in bilayers formed from a rhodopsin proteolipid which had been purposely bleached, the membrane conductance showed clearly defined fluctuations indistinguishable from those displayed in bleached membranes after the dark to light transition (see below).

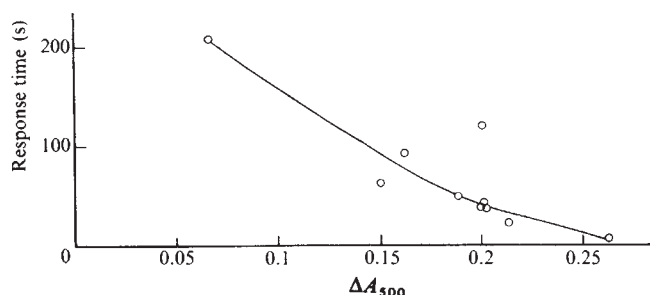
This conductance pattern can be recorded in symmetrical media containing either KCl or NaCl as the electrolyte. Ca²⁺ enhances the conductance changes recorded in planar bilayers¹⁰: in the presence of Ca²⁺, the frequency of the conductance fluctuations and the highest levels of conductance reached upon illumination are higher than in its absence.

A distinct latency period between illumination and onset of conductance changes was characteristic in all experiments. Figure 2 illustrates the dependence of the bilayer response time on the rhodopsin concentration present in the proteolipid. Although the total amount of rhodopsin in the proteolipid is known, we have no way of determining the rhodopsin concentration in the bilayer. The latency period varied from 3–200 s and was shorter the higher the rhodopsin content in the proteolipid extract.

In experimental conditions of a steady-state voltage of 20 mV, and a 0.2 M NaCl, 1 mM CaCl₂ solution, three main types of transitions appeared, with amplitudes of about 2, 4 and $6 \times 10^{-10} \Omega^{-1}$ (Fig. 3). The distribution of G_m transitions was equivalent in dark and bleached membranes (Fig. 3). Thus, illumination does not change the fluctuation pattern, it merely increases the absolute conductance and the frequency of fluctuations. A current trace showing the three transitions is shown in Fig. 4. These results indicate the light-induced formation of a channel by rhodopsin.

The individual conductance transitions were ohmic up to 100 mV (Fig. 5). In contrast, the macroscopic G_m was not ohmic but voltage dependent. This is illustrated in Fig. 6: at voltage steps of ≤ 20 mV, G_m displays a steady value with fluctuations of the established magnitude; at voltages greater than about 20 mV the initial G_m relaxes to lower steady-state values. The curve in Fig. 6 shows a region of low voltage sensitivity between 0 and 20 mV. At higher voltages a steep drop of the normalised conductance occurs reaching almost zero. This behaviour establishes the voltage sensitivity of the rhodopsin channel, that is, at high voltage, the channel closes.

Fig. 2 Dependence of the bilayer response time on the rhodopsin content of the proteolipid. Bilayers were prepared from ether proteolipids exhibiting different ΔA_{500} . The time after bleaching required to detect a definite conductance increment is presented in the ordinates. Other experimental conditions as Fig. 1.



As seen from the insets of Fig. 6 the time constants τ_c of closing are in the order of seconds: for example, at a clamping potential of 50 mV the main part of the closing process occurs with a $\tau_c \approx 3$ s. Fast switching the voltage (time constant ~ 30 ms) to zero or inverting the polarity opens the channel. Therefore, the time constant of channel opening is much faster than that for channel closing.

The situation illustrated so far is characteristic of media containing either NaCl or KCl (and 1 mM CaCl_2). But we also made conductance measurements with two other cations— Ca^{2+} and TEA^+ (tetraethylammonium). With both ions in symmetrical 0.2 M solutions, voltage-dependent G_m and G_m fluctuations were seen.

Concentration potential measurements performed in bilayers separating two compartments with different salt

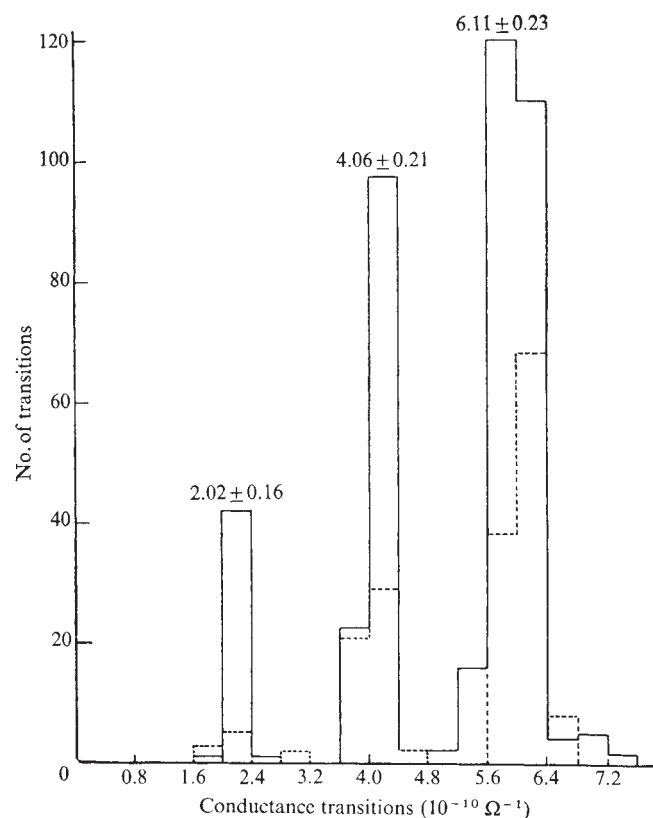


Fig. 3 Histogram of the conductance transitions in dark and bleached states. Only well defined current transitions observed at the steady state voltage of 20 mV in 0.2 M NaCl, 1 mM CaCl_2 , 10 mM imidazole pH 7 of different membranes are collected for the histogram. Two sets of data are presented: transitions in dark (---), and in bleached (—) membranes. Fluctuations of $\leq 0.8 \times 10^{-10} \Omega^{-1}$ and conductance bursts $\geq 8 \times 10^{-10} \Omega^{-1}$ are not included. The ether proteolipids used for the bilayer membranes exhibited ΔA_{500} of $0.17 < \Delta A < 0.26$. Other experimental conditions as for Fig. 1.

activities, showed that G_m is cation selective. Transference numbers for H^+ , K^+ and Ca^{2+} , calculated from the membrane potentials, are 0.85, 0.83 and 0.9 respectively.

The proteolipid in the organic solvent solution contains, in addition to spectrally native rhodopsin, residual detergent, free retinal, and opsin. It is reasonable to ask which of these four components are responsible for the observed effects. Appropriate control experiments were therefore performed: light-induced conductance increases and current fluctuations have been observed with bilayers formed from monolayers of retinal rod disks spread by Trurnit's method, and in this situation, of course, detergent is totally absent^{12,15}. Unfortunately, this system cannot be used

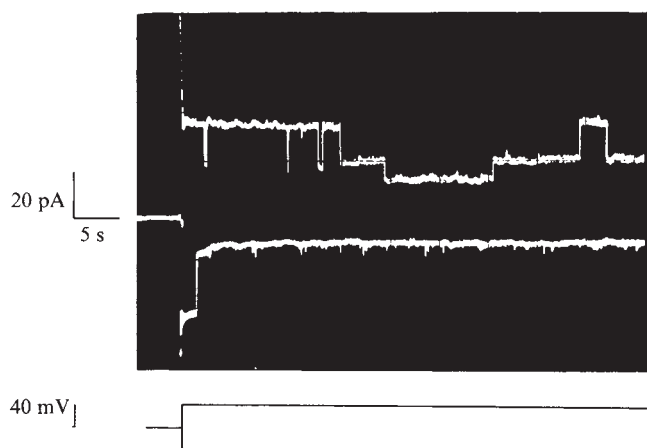
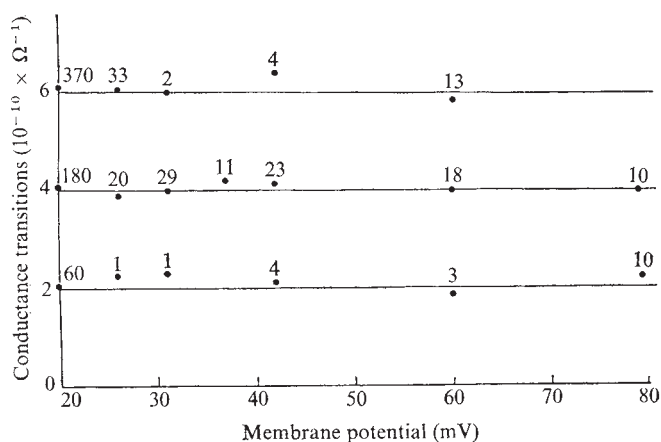


Fig. 4 Membrane current at voltages of +40 and -40 mV from a bleached rhodopsin bilayer. The initial current transient corresponds to the capacitive component. The transition of 2 and $4 \times 10^{-10} \Omega^{-1}$ can be calculated from the positive current record. A transition of $6 \times 10^{-10} \Omega^{-1}$ is recorded in the negative current trace. The G_m fluctuations are not due to steps in bilayer breakdown: they can be recorded even at 10 mV in membranes which are stable up to 3 h. The bilayer was prepared as for Fig. 1.

routinely because of the difficulty in bilayer formation. Alternatively, the recombination of retinal rod disks with phospholipids by sonication allows the extraction of a rhodopsin proteolipid into the solvent with an efficiency comparable with that of the second-ether-extract from CTAB solubilised rhodopsin. It is clear, therefore, that this proteolipid has never been in contact with detergent. Planar bilayers formed from this proteolipid display the same overall behaviour described before in detail: this rules out the participation of the detergent and establishes that rhodopsin is the species responsible of the membrane conductance. The experiments with this proteolipid, which we term 'instant proteolipid', because it speeds up the preparation by obviating the solubilisation step, will be reported in detail elsewhere (in preparation). Another control was done with bilayers formed from proteolipids of opsin (prepared by removal of retinal from bleached rhodopsin¹⁶) which had high conductance ($G_m \sim 10^{-9} \Omega^{-1}$), displayed conductance fluctuations of 2, 4 and $6 \times 10^{-10} \Omega^{-1}$, exhibited a voltage dependent reduction of G_m and, in their overall behaviour, were indistinguishable from those prepared from

Fig. 5 Voltage-dependence of the conductance transitions. The small numbers represent the number of events. Note that the number of transitions decreases as the voltage increases. Experimental conditions as for Fig. 1.



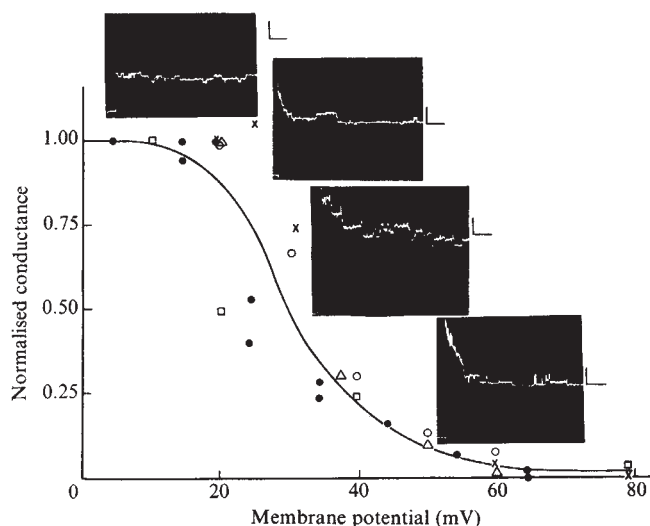


Fig. 6 Voltage dependence of the normalised membrane conductance (G_m at given voltage/ G_m at zero voltage). The figure contains insets of photographs of the current relaxation for voltage steps of 20, 31, 39 and 60 mV, respectively. The lowest line in the photographs represents the baseline at zero current. The initial current peak corresponds to a capacitive transient associated with the onset of the voltage. The vertical scale bars to the right of the photographs represents the current calibration: 50 pA; the horizontal scale bars are the time calibration, 2 s. Two sets of data are presented in the plot: the first set is obtained from four highly conducting membranes ($G_m > 16 \times 10^{-10} \Omega^{-1}$) ($\bullet$, $\circ$, $\times$, $\square$); for the second set the four different membranes of low conductance ($G_m \leq 16 \times 10^{-10} \Omega^{-1}$) ($\triangle$) were treated as a single membrane.

bleached rhodopsin. As a further control, proteolipids prepared from thermally denatured retinal-free opsin yielded bilayers with low conductance ($G_m < 10^{-10} \Omega^{-1}$); occasional periods of high frequency noise were observed, but these without discernible current step fluctuations. It seems clear, therefore, that the bilayer conductance and its fluctuations are also associated with the presence of opsin. Thus, the variable levels of conductance given by control (that is, dark) rhodopsin-proteolipid bilayers must reflect the presence of variable amounts of bleached rhodopsin (that is, opsin). As noted above, however, whatever the control level of conductance, the effect of light is usually a displacement of the conductance to higher levels.

The planar bilayer experiments have been repeated with proteolipids made from many (>50) different rhodopsin preparations. Light responses of the type shown in Fig. 1 were seen in 30 of the 60 rhodopsin membranes displaying G_m fluctuations. Further technical and analytical refinements should facilitate a more precise characterisation of the bilayer ion-specificity, the kinetics of light-activated responses and light-energy dependence, which could eventually match with the characteristics of photoreceptor membranes².

The light-induced transmembrane channel

It has been shown that light increases the membrane permeability of rhodopsin-containing bilayer membranes. The conductance fluctuations observed in the rhodopsin planar bilayers are of the type induced in lipid bilayers by molecules known to form transmembrane channels^{17,18}. Further, the conductance transitions of the order of $10^{-10} \Omega^{-1}$ indicate a univalent ion flux of 10^8 particles per s for a 100-mV driving force. This calculated flux is higher by four orders of magnitude than the maximal transport rate of an ion-carrier, such as valinomycin, but is the expected value for a channel^{17,18}.

The evidence, taken altogether, establishes that the effect of bleaching rhodopsin in bilayer membranes is to induce the formation of a transmembrane channel. Considering a

channel conductance of $4 \times 10^{-10} \Omega^{-1}$, it is possible to make an order of magnitude estimation of its smallest diameter. Assuming the channel to be a cylindrical pore filled with the bathing electrolyte of bulk conductivity (0.2 M NaCl) and having a length comparable with the membrane thickness of 30 Å, its diameter would be 9 Å (ref. 10). If the channel would be stringently cation selective and using only the Na^+ bulk conductance, its diameter is calculated to be 14 Å. These values seem to be reasonable estimates since the crystal diameter of TEA^+ is about 8 Å (ref. 19) and TEA^+ can pass through the channel. Hydrogen-tritium exchange measurements in rod disk membranes are consistent with the formation of a hydrophilic channel of about 10–15 Å in diameter²⁰, though the data have been recently questioned²¹. Complementary experiments with rhodopsin incorporated into lipid vesicles showed that exposure of the vesicles to light increases the permeability to Na^+ , Cs^+ , Ca^{2+} , glycerol and glucose but not to Cl^- , sucrose and inulin (in preparation). These results indicate that light induces the formation of a wide permeability pathway with a cutoff diameter of about 10 Å. The wavelength dependence of this phenomenon together with a set of control experiments that exclude the involvement of retinal and residual detergent establish that the light response can be accounted for by rhodopsin bleaching¹⁴.

Aggregation can explain the latency of the light response

An immediate obvious difference between the light-induced changes we record *in vitro* and the situation *in vivo* is the relatively long lag period between applications of light and the photoresponse: seconds compared with milliseconds *in vivo*². This difference can be explained by postulating that rhodopsin bleaching changes the permeability by a diffusion limited aggregation process. Such a cooperative process between rhodopsin molecules has been thought to be involved in vertebrate scotopic vision²².

Different models have been considered to explain the experimental observations of the channel and of channel formation in the planar bilayer. A unimolecular channel would be the simplest model. This model suffers from the difficulty of explaining the lag period of the conductance increase after bleaching. The difficulty arises from the assumption that metarhodopsin II, which is produced in milliseconds at room temperature³ (the experimental condition of the planar bilayers), is a conducting species—as seen from the vesicle experiments^{11,14}.

The latency period could be perhaps best explained by a channel formed from rhodopsin units. These aggregating molecules would form after bleaching, and would have to find each other by diffusion in the plane of the membrane. Since the membrane contains only few channels, and thus few aggregating species (assuming a homogenous distribution of rhodopsin), then the observed latency is actually shorter than expected on the basis of unrestricted diffusion. For example, with a diffusion constant of $\sim 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ (ref. 23) for rhodopsin in the bilayer, the molecule travels only 10^{-4} cm in 100 s, that is 1/100 of the actual membrane diameter. To overcome this discrepancy one has to assume that the diffusion occurs in a severely constrained domain. Indeed, examination of the surface layer from which the membranes are formed by phase contrast microscopy, shows patches of the size in question, that is, 10^{-4} cm diameter; they appear white, time stable and probably contain opsin. In contrast, in the disk membrane the protein-protein distance is only tens of Ångströms and so the diffusion-dependent delay is much less than 1 ms, and has no effect on the physiological response time.

Further support for this proposal is that the lag time decreases as the rhodopsin concentration in the proteolipid increases, as seen from Fig. 2.

Once the channel is formed it is fixed and stable. Its

voltage sensitivity cannot be explained by dissociation and subsequent diffusion, since a voltage step to the inverted polarity restores the high conductance state in less than 0.1 s, a time which is considerably shorter than that required for the formation of the channel.

Our favoured model, therefore, is that of a fixed conducting aggregate formed from subunits.

Proposed model of visual excitation

What is the link between the photochemistry of rhodopsin and the bilayer permeability response? The photoisomerisation of the retinal chromophore in rhodopsin triggers transitions in the protein through a sequence of conformational states. The metarhodopsin I to metarhodopsin II transition is the last step in the bleaching sequence which is fast enough to be involved in the transduction process^{2,3}. Since the light-induced permeability in rhodopsin vesicles is elicited at 3 °C, metarhodopsin II may be the first conducting (aggregating) form of rhodopsin.

We have detected capacitative photocurrents in rhodopsin layers from which the bilayers are formed; these arise from charge displacements in rhodopsin as it bleaches²⁴. The photocurrents occur in milliseconds and thus precede the formation of the channel. In analogy to the sodium channel of nerve membranes, where a capacitative—'gating'—current is associated with its opening and closing²⁵, it is tempting to suggest that the measured photocurrents represent the gating current of the rhodopsin channel²⁴.

Irrespective of whether the channel is formed from one or several rhodopsin molecules, it is evident that the amplification gained on allowing the passage of more than 10⁶ ions per s per absorbed photon, is indeed, enormous^{1,2,4,8}.

In vertebrate rods, rhodopsin is located in the intracellular disks and the photoinduced reduction of the 'Na⁺-dark current' occurs in the plasma membrane: a connecting signal between the two membranes is required^{2,3}. Calcium has been postulated as the transmitter²: cytoplasmic Ca²⁺ activity increases during illumination because of a light-induced permeability of the disk membrane to Ca²⁺; Ca²⁺ regulates the 'Na⁺-dark current' by binding reversibly to the plasma membrane sites through which Na⁺ flows into the outer segment. In addition, a Ca²⁺-ATPase has been described in rod outer segments²⁶ and in purified disk membranes²⁷. Based on our findings a model of visual phototransduction can be proposed: in the dark, the channel is closed and the ATPase would pump Ca²⁺ into the disks; on bleaching, the channel

would form and allow Ca²⁺ to diffuse down its concentration gradient. The diffusion of Ca²⁺ would change the disk membrane potential to the value (threshold) at which the channel closes. The channel closure, the photoregeneration of rhodopsin, as well as the activity of the pump, restore the membrane to the conditions existent in the dark. Thus, a light-induced and voltage-sensitive channel constitutes an efficient phototransducer that utilises the well established principles which govern membrane excitability and considers the neural origin of retinal photoreceptors²⁸. There is no evidence at present to extend this scheme to the invertebrate photoreceptors where the situation seems more complex^{2,3}.

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X-ray study of the lithium complex of NAD⁺

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The Li⁺·NAD⁺ complex exists as a 'dimer' of two molecules arranged head-to-tail with Li⁺ coordinated tetrahedrally to adenine N(7) and three pyrophosphate oxygens. Adenine is stacked intermolecularly on nicotinamide. The conformation of NAD⁺ is 'extended' and similar to that found in holoenzyme complexes. This is in contrast to the 'folded' structure proposed from spectroscopic studies.

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BIOLOGICAL redox reactions are catalysed by enzymes requiring cofactors which receive hydride or electrons from one substrate (oxidation) and donate them to another (reduction). Three main groups of enzymes can be distinguished: those containing the (dissociable) coenzymes NAD⁺ or NADP⁺, those with the (non-dissociable) prosthetic groups FAD or FMN, and the cytochromes. The largest group is that of NAD⁺- or NADP⁺-dependent dehydrogenases of which over 150 have so far been studied^{1–3}.

NAD⁺ was first discovered in 1904 by Harden and Young. It was isolated in 1931 by von Euler and co-workers

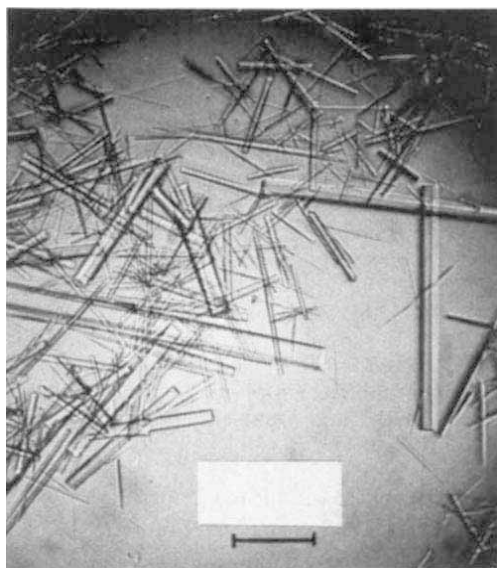


Fig. 1 Crystals of the $\text{Li}^+\cdot\text{NAD}^+\cdot 2\text{H}_2\text{O}$ complex.

and its chemical structure was elucidated in 1936 by Warburg and Christian¹. Since then, numerous spectroscopic studies such as fluorescence^{4,5}, circular dichroism⁶ and nuclear magnetic resonance (proton, C-13 and P-31)⁷⁻¹⁴ have been performed on NAD^+ , NADP^+ and synthetic analogues^{2,15-17} in order to establish the three-dimensional structure. It was found that in aqueous solution, NAD^+ occurs in a 'folded' conformation with the two heterocycles forming an intramolecular stack. Depending on the stack (adenine on nicotinamide or vice versa), right- and left-handed helices result which are assumed to be in a rapid equilibrium with an intermediate 'extended', non-stacked form (see ref. 10 for review).

More direct information about the structure of NAD^+ was obtained from X-ray crystallographic data of its holo-enzyme complexes with lactate dehydrogenase (LDH), soluble malate dehydrogenase (s-MDH) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and liver alcohol dehydrogenase (LADH) (ref. 1). All four enzymes bind NAD^+ in a related 'extended' conformation and not in the folded form proposed for NAD^+ in solution. These analyses also revealed that NAD^+ can bind with the nicotinamide residue either in the *syn* or *anti* orientation, depending on the enzyme, and thus hydride transfer to the C(4) position can occur from both sides of the pyridine ring (see ref. 2 for classification of A and B dehydrogenases).

Attempts to crystallise NAD^+ date back to 1957 but

until this present investigation no specimens suitable for an X-ray analysis had been reported¹⁸. In this paper we describe our crystallographic studies carried out on the $\text{Li}^+\cdot\text{NAD}^+$ complex.

Experimental procedure

For crystallisation, 1 g lyophilised NAD^+ free acid was dissolved in 0.75 ml water and adjusted to pH 4.0 by slow addition of 1.3 ml 1 N LiOH. Then 2 ml methanol were added and the solution was kept at 20–25 °C for 30 h. The crystallised product (Fig. 1) was filtered off, washed twice with acetone and dried over P_2O_5 in a vacuum. Yield was 0.9 g (90%) $\text{Li}^+\cdot\text{NAD}^+\cdot 2\text{H}_2\text{O}$, and analysis showed NAD^+ (enzymatic with alcohol dehydrogenase, ADH) at 93.5%; water (by Karl Fischer method), 5.5%; lithium (flame photometric), 1.1%; Fp uncorr., 203–204 °C. $\text{Li}^+\cdot\text{NAD}^+\cdot 2\text{H}_2\text{O}$ is free of inhibitors for the LDH-reaction and the test according to Amador¹⁹ gives 10–15% better results than commercially available ' NAD^+ free acid' preparations.

A crystal of dimensions $0.025 \times 0.025 \times 0.2$ mm was isolated from the methanolic solution, sealed with some mother liquor in a capillary and used for all X-ray studies. Space group and cell dimensions are $\text{P}2_12_1$ and $a=10.073(3)$ Å, $b=15.839(4)$ Å, $c=17.821(4)$ Å. Intensity data were collected in the $2\theta/\omega$ scan mode by means of an automated STOE four-circle diffractometer using Ni-filtered $\text{CuK}\alpha$ radiation. Owing to the rapid decrease of intensities with increasing glancing angle θ , only a limited data set corresponding to a resolution of 1.09 Å ($\theta_{\text{max}}=45^\circ$) could be measured. Out of the 1365 data, 338 were below the 3σ threshold and considered as unobserved.

The structure was solved by heavy atom and direct methods employing the multisolution tangent formula approach MULTAN²⁰. Although the most consistent phase set derived with the 450E's greater than 1.01 had a rather 'high' Karle *R*-factor of 50.4%, the resulting *E*-map showed maxima which could be identified as atoms of the pyrophosphate group and of the adenine heterocycle. The rest of the structure was derived using Fourier techniques. Atomic parameters were refined by full matrix least squares cycles with assignment of anisotropic temperature factors to P and O atoms. The Li^+ ion and some hydrogen atoms could be located from a difference Fourier synthesis at the end of the refinement which finally converged at $R=10.0\%$ for the observed data.

NAD⁺ structure

As shown in Fig. 2, NAD^+ is composed of adenylic acid and nicotinamide-5'-ribonucleotide fused together in a head-to-head orientation through a pyrophosphate linkage.

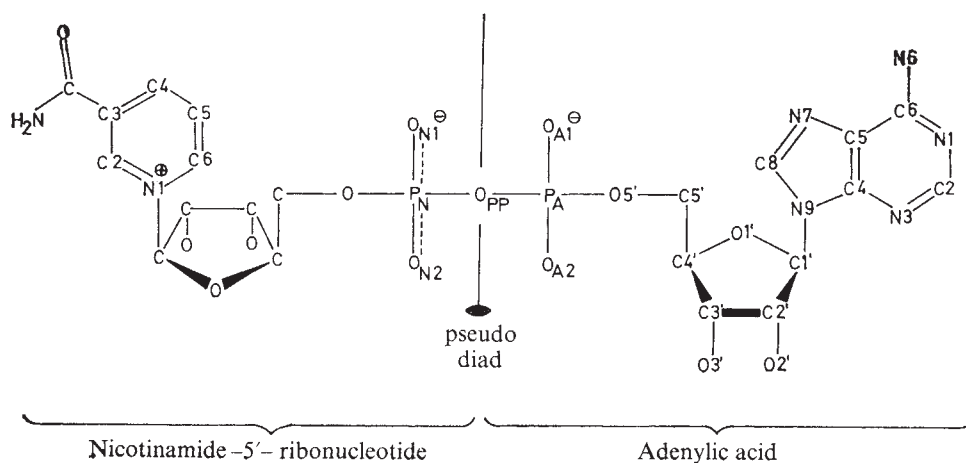


Fig. 2 Chemical structure of NAD^+ and atom numbering convention used. The pseudo diad relating the two ribosephosphate moieties is indicated.

From a structural point of view, NAD^+ could be diad symmetrical (neglecting the difference in heterocycles) with the diad passing through the pyrophosphate O(PP) oxygen atom. In the Li^+NAD^+ complex, however, asymmetry is introduced by two Li^+ cations liganded to the same NAD^+ molecule, one attached to adenine N(7) and a free oxygen atom of the P_N phosphate while the second Li^+ occupies the other free oxygen of this phosphate group and one free oxygen of the P_A phosphate (Fig. 3). In this complex the NAD^+ molecule does not exist in the 'folded' conformation with adenine and nicotinamide heterocycles stacked with each other but rather assumes an 'extended' conformation with the planes passing through the heterocycles in an almost perpendicular arrangement.

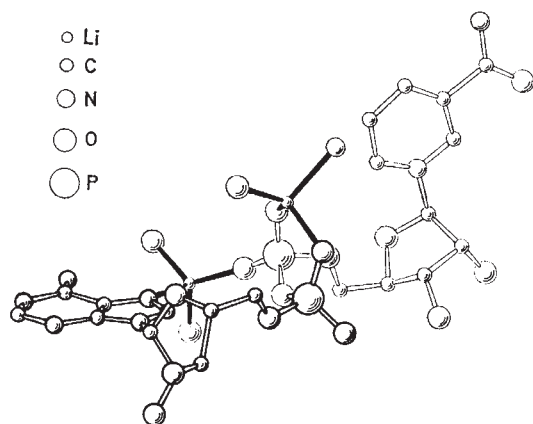


Fig. 3 Molecular structure of the Li^+NAD^+ complex. Coordination to two Li^+ -ions is marked by filled bonds, parts of the molecule nearer to the observer (adenosine moiety) are indicated by heavier lines. Note that adenine and nicotinamide planes are almost perpendicular to each other.

Conformation of the two nucleotides

For 5'-ribonucleotides the energetically most favoured conformational parameters can be summarised briefly as follows; the sugar is puckered C(3')-*endo* or C(2')-*endo*, the heterocycle is in an *anti* position about the glycosyl C(1')-N bond, the orientation about the C(4')-C(5') bond is *gauche*, *gauche* locating O(5') 'above' the ribose and, finally, the phosphate group is *trans* to C(4') (refs 21-23).

In Li^+NAD^+ the ribose bearing the nicotinamide residue is puckered in a C(3')-*endo* envelope form while in the adenosine part the ribose occurs in a C(2')-*endo* twist conformation, probably a consequence of the particular geometrical requirements imposed on the molecule by the coordination of Li^+ to both adenine N(7) and one free oxygen atom of the phosphate group P_N .

In both nucleotides the heterocycles are in an *anti* orientation with torsion angles O(1')-C(1')-N(9)-C(8) of 52° for adenosine and O(1')-C(1')-N(1)-C(6) of 15° for nicotinamide riboside. In the latter case, it seems that the positive charge at N(1) modifies the C(1')-N bond distance, its value of 1.55 Å being significantly longer than the 1.47 Å generally observed in pyrimidine nucleosides, but it has no obvious influence on the nucleotide conformation. The orientations about the C(4')-C(5') bond and about the C(5')-O(5') bond in both nucleotides correspond to those usually considered as preferred, that is, *gauche*, *gauche* and *trans*, respectively.

As in other N(1) substituted nicotinamide moieties, the amide group in NAD^+ is nearly coplanar with the heterocycle, the C-NH₂ bond being *cis*-planar with the C(3)-C(4) bond²⁴⁻²⁶. In the unsubstituted nicotinamide²⁷, however, C-NH₂ is *trans*-oriented to C(3)-C(4).

The pyrophosphate linkage

So far, four other crystal structures containing nucleotide pyrophosphate linkages have been reported, ATP, ADP, CDP and CDP-choline²⁸⁻³⁰. Details of the pyrophosphate group have also been described for the symmetrical di-β-naphthyl-pyrophosphate³¹ and for thiamine-pyrophosphate³² and its hydrochloride³³. In all of these molecules the P-O distances in the P-O-P fragments differ significantly (more than three e.s.d.s) from each other. Although NAD^+ possesses a theoretical twofold symmetry about the pyrophosphate oxygen atom O(PP), the P-O(PP) distances differ greatly with a P_A -O(PP) bond length of 1.56 Å and P_N -O(PP) of 1.66 Å. This is attributable to the asymmetrical Li^+ coordination. As with other pyrophosphates, the P-O-P angle is 133° and this relatively obtuse angle allows eclipsed conformation about the P-O(PP) bonds (torsion angles $\pm 120^\circ$) which are normally avoided. There is thus an appreciable flexibility about the pyrophosphate linkage as demonstrated by the almost evenly filled conformational wheel in Fig. 4. On the other hand, the torsion angle about the 'exo-pyrophosphate' link O(5')-P is generally limited to (+) *gauche* with an exceptional -124° for the adenosine part in NAD^+ , probably a result of the steric peculiarities forced by the Li^+ coordination.

Li⁺ cation coordination

One of the most interesting aspects of this crystal structure concerns the coordination of the Li^+ cation. The four tetrahedrally arranged ligands N(7), O(N1), O(N2), O(A1) (In this nomenclature, unesterified phosphate oxygen atoms

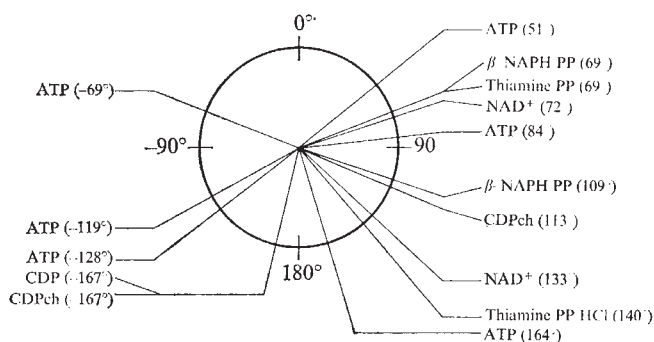


Fig. 4 Conformational wheel for torsion angles O-P-O-P about the pyrophosphate P-O linkage. Note that essentially all angles do occur except those with *cis*-planar arrangement (near 0°). CDPch, CDPcholine; β-Naph PP, di-β-naphthyl-pyrophosphate; thiamine PP, thiamine pyrophosphate, and thiamine PP-HCl, its hydrochloride derivative.

are indexed 1 and 2 if, looking along the O(5')→P bond, they are at 120° and 240° clockwise rotation from the pyrophosphate link P-O(PP)) are at distances of 2.13 Å, 1.86 Å, 1.92 Å and 1.88 Å from Li^+ respectively and are provided by two different NAD^+ molecules held together by the Li^+ cation in a head to tail orientation (Fig. 5). One NAD^+ molecule donates the two unesterified pyrophosphate oxygen atoms O(A1) and O(N1) and the other the adenine N(7) and the phosphate oxygen atom O(N2). A similar situation was found in the Na_2ATP complex²⁷ where the adenine N(7) atom is bridged with unesterified oxygen atoms of the γ-phosphate group, the coordination to the more remote γ-phosphate (instead of β-phosphate) probably reflecting the different radii of the cations: 0.68 Å for Li^+ against 0.97 Å for Na^+ . In this respect one might expect that Mg^{2+} with a radius of 0.66 Å would form cation-nucleotide complexes similar to those of Li^+ and not Na^+ or K^+ .

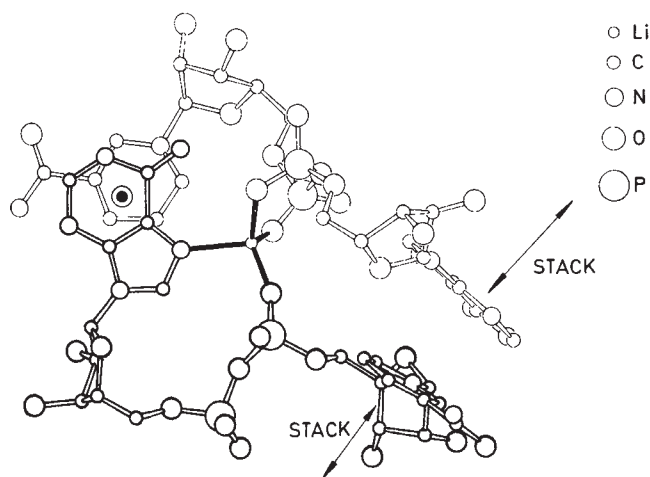


Fig. 5 The $\text{Li}^+ \cdot (\text{NAD}^+)_2$ dimer projected along the intermolecular stack indicated by $\odot$. Stack formation with other, symmetry related molecules in the crystal lattice are marked by arrows. One of the two NAD^+ molecules is drawn in heavier type for clarity.

Adenine and nicotinamide intermolecular stacking

Figure 5 demonstrates that the two NAD^+ molecules linked by one Li^+ cation interact through stack formation ($\sim 3.4 \text{ \AA}$) between the adenine coordinated to Li^+ and the partner nicotinamide. The partner adenine forms a stack with a symmetry related NAD^+ molecule to which it is bonded through another Li^+ bridge and so on. In this stack, the adenine N(6) amino group is close to the pyridine nitrogen atom, and the glycosyl linkages are oriented anti-parallel (Fig. 5), features also observed in the 1-methylnicotinamide-aden-9-yl acetate cocrystallisate²⁶ but not in NAD^+ analogues with methylene chains replacing the ribotides^{34,35}. A similar arrangement of heterocycles was also proposed for the intramolecular stack in the 'folded' NAD^+ in aqueous solution^{7,10}.

Interaction between NAD^+ molecules in crystal lattice

As mentioned above, the NAD^+ molecules form Li^+ -coordinated, stacked 'dimers' which complex and stack with symmetry related neighbours, thus generating columns building up the crystal lattice. 'Empty' spaces between these columns are filled with two water molecules per NAD^+ , assisting in the formation of a complicated hydrogen bonding network (to be discussed elsewhere).

In aqueous solution, the 'polymeric' $\text{Li}^+ \cdot \text{NAD}^+$ complex will dissociate into electro-neutral $\text{Li}^+ \cdot \text{NAD}^+$ units with Li^+ coordinated intramolecularly either to N(7) and phosphate oxygen O(N2) or to the free pyrophosphate oxygen atoms O(A1) and O(N1), the remaining two coordination sites of Li^+ being satisfied by water molecules. Li^+ will be distributed between these two positions according to the respective association constants.

Conclusions

It is of particular interest to compare the structure of the $\text{Li}^+ \cdot \text{NAD}^+$ complex with those derived from the numerous spectroscopic investigations carried out in the past two decades and with the conformation of NAD^+ when it forms holoenzyme complexes with dehydrogenases.

Conformation of NAD^+ devoid of Li^+

The $\text{Li}^+ \cdot \text{NAD}^+$ complex represents only one possible low-energy structure of the NAD^+ molecule and with other

(larger) cations unable to coordinate with adenine N(7) and the P_N phosphate oxygen atoms simultaneously, geometries different to those observed here might be obtained. Some features of general importance will remain such as the preferred conformations of the two individual nucleotides but the orientation about the pyrophosphate linkage could change because of the flexibility about the $\text{P}-\text{O}(\text{PP})$ bonds as demonstrated in Fig. 4.

Correlation with results from spectroscopic data

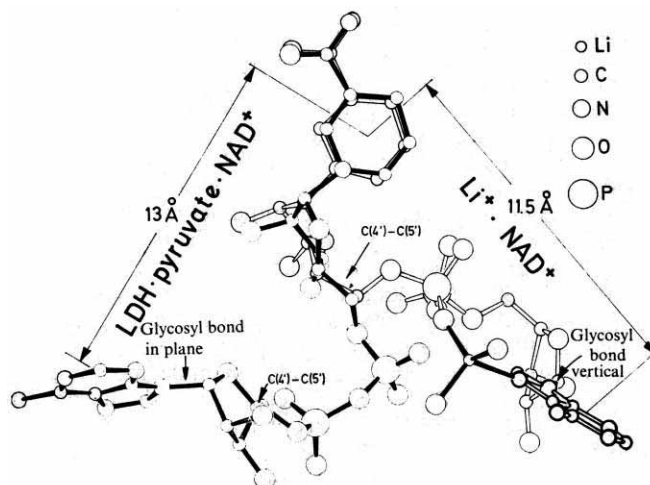
The most conclusive results on the conformation of NAD^+ in aqueous solution have been derived from NMR data. Although the proposal of two helical, 'folded' structures of NAD^+ was criticised in favour of the extended one¹¹, it seems to be corroborated by the numerous data. The helical geometry is even preserved when NAD^+ dimerises at higher concentrations¹⁰. From stereochemical considerations, a 'folded', helical structure for NAD^+ is feasible only if no chelation exists between adenine N(7) and the P_N phosphate group. Owing to the flexibility of the pyrophosphate linkage (Fig. 4), the respective torsion angles can be adjusted to form an intramolecular stack described in Fig. 5 for the $\text{Li}^+ \cdot \text{NAD}^+$ complex; N(6) of adenine is close to N(1) of nicotinamide and the glycosyl linkages are in nearly anti-parallel orientation. It should be noted that such a folding of the NAD^+ molecule can be achieved with the preferred conformations of both nucleotides fully maintained ($\text{C}(2')$ or $\text{C}(3')$ -endo; *gauche*, *gauche*; *anti*).

In the light of the structure of the $\text{Li}^+ \cdot \text{NAD}^+$ complex with intermolecular chelation and dimer (stack) formation, however, it seems necessary to look at the interpretation of the spectroscopic data more critically, especially since in most previous work the nature of the counter ion has not been considered.

NAD^+ conformation on binding to enzymes

The crystal structures of enzyme- NAD^+ complexes have been described for LDH, LADH, GAPDH and s-MDH¹. In

Fig. 6 Structures of the NAD^+ molecules in the Li^+ complex (open bonds) and in the ternary complex $\text{LDH} \cdot \text{pyruvate} \cdot \text{NAD}^+$ ³⁷ (solid bonds; NAD^+ in the holoenzyme complexes has a similar conformation as in the ternary complex). Both molecules are projected on the plane through the nicotinamide heterocycle for easy comparison. Owing primarily to the different conformations about the $\text{C}(4')-\text{C}(5')$ bonds indicated by arrows, the adenosines are in different orientations relative to nicotinamide although the distances between the heterocycles are comparable. Note that the adenine planes are nearly perpendicular to the nicotinamide planes and that they are related by a 90° rotation: in $\text{Li}^+ \cdot \text{NAD}^+$ the glycosyl $\text{C}(1')-\text{N}$ bond is nearly vertical to the paper while it is in the paper for the ternary complex.



all of these enzymes, the nucleotide binding sites are geometrically related³⁶ and the bound NAD⁺ molecules display similar conformational features as shown in Fig. 6 for the ternary LDH·NAD⁺·pyruvate complex^{37,38}. It is striking to find that the intramolecular distances between the adenine and nicotinamide heterocycles, 11.5 Å in Li⁺·NAD⁺ and 13 Å in the ternary complex are comparable and that in both NAD⁺ molecules the conformation is such that the planes comprising adenine and nicotinamide are nearly perpendicular to each other. The form of NAD⁺ as found in the Li⁺ complex can therefore be considered as that form recognised by the enzyme before complex formation while the 'folded' conformation with parallel heterocycles about 3.4 Å apart seems to be too different to be directly involved in this process.

The conformations about the C(4')–C(5') bonds for NAD⁺ in the holoenzyme and Li⁺ complexes, however, constitute a significant difference. While for the Li⁺·NAD⁺ complex the preferred *gauche*, *gauche* conformation is found, it is *trans gauche*, for both nucleotides in the holoenzyme NAD⁺ molecule, corresponding to a high-energy state³³. This conformation has never been observed for any 5'-ribonucleotide except for the modified 6-azauridine-5'-phosphate (ref. 39).

Several factors can force such a drastic conformational change of a molecule, primarily the dehydration of NAD⁺ when diffusing from the aqueous solution to the enzyme surface. Further, the liganded cation will be expelled and replaced by positively charged groups of amino acid side chains lining the NAD⁺ binding site. These groups not only compensate for the positive charge but they can also form hydrogen bonds to phosphate oxygen atoms, to ribose hydroxyl groups and to the nicotinamide residue and, further, the adenine heterocycle fits into a 'hydrophobic' pocket^{1,36}. Together, these small individual forces can obviously induce a significant conformational change of NAD⁺.

Similarities of NADP⁺ and NAD⁺ conformations

NADP⁺ differs from NAD⁺ by an additional phosphate group attached to O(2') of the adenosine part. Because this phosphate is fairly remote from the pyrophosphate linkage, these two groups will not interact directly or by means of a chelated cation, at least as long as the two nucleotides

themselves maintain their preferred conformations. On the other hand, the adenine N(3) atom would be in a suitable position to be liganded by a cation connected with the oxygen atoms of the O(2')-phosphate.

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letters to nature

Evidence for a 581-d modulation in the pulse period of 3U0352 + 30

We have shown¹ that 3U0352+30 is an X-ray pulsator with a period of 13.9 min. There is also evidence for a modulation in the X-ray flux at 22 (or possibly 11) h but this phenomenon is less well-defined¹. The X-ray source has been identified with the 6 mag Be star X Per on the basis of position (ref. 2 and references therein) and interstellar absorption column³. Neither modulation has yet been detected in the broad-band optical flux^{4,5}, though there is unconfirmed evidence for a 7-min periodicity in a narrow band centred on 4,686 Å (ref. 6). Hutchings *et al.*⁷ have measured periodic shifts in the profiles of the Balmer and He I spectral lines of X Per with amplitudes of ~ 60 and 10 km s⁻¹ respectively which they interpret as due to orbital motion with a period of 581 d. They suggest that the Balmer

line shifts represent the underlying motion of X Per. This implies a mass for the unseen companion of tens of solar masses and indicates that it might be a black hole⁸. The necessity of reconciling all the X-ray and optical data has caused this interpretation to be questioned^{8,9}. Henrichs and Van den Heuvel⁸ suggest that the 581-d periodicity might reflect the apsidal motion of a neutron star in a 22-h orbit about X Per, while Milgrom⁹ postulates that the true 581-d orbital velocity is given by the He I absorption lines in the spectrum (velocity amplitude ~ 10 km s⁻¹), implying an unseen companion of only ~ 2M_⊙.

Copernicus has observed 3U0352+30 on a total of eight occasions between October 1972 and February 1977 as summarised in Table 1. We have searched five days of data from the observing run in February 1976 for a modulation in the pulsation period due to 22-h orbital motion. For a trial orbital period X the data were multiplied by a masking function F,

such that $F = 1$ for $0.0 < \phi_x < 0.33$ and $F = 0$ for $0.33 < \phi_x < 1.0$, where ϕ_x is the phase of the trial period. The 13.9 min. modulation period is then measured by power spectrum analysis¹. The mask is shifted by 0.16X and the process repeated until a complete cycle is covered. In this way an upper limit has been set to $V \sin i$ of 200 km s⁻¹ for periods in the range 11–60 h. In Table 2 we give upper limits to the orbital inclination, i , for periods of 11.2, 22.4 and 44.8 h assuming a $20M_\odot$ primary and a $2M_\odot$ secondary in a circular orbit.

The mean heliocentric period of the 13.9 min modulation measured from each of the eight Copernicus observing runs is given in Table 1. The technique used to determine these values is as described in ref. 1 except that the uncertainties in the periods have been determined using the formula given by Middleditch and Nelson¹⁰. Numerical simulations on the data show that the confidence level of the uncertainties derived in this way is better than 99%. Table 1 shows that there are significant differences in the periods derived. We have investigated the possibility that these fluctuations are related to the 581-d optical period by fitting a function of the form

$$P = P_0 + P't + A \sin(2\pi(t - t_0)/P_{orb})$$

where P_0 is the pulse period on JD 2441317.5 (0 h, 1st January 1972) and P_{orb} and t_0 are the period and epoch of a long term modulation. P_0 , P' , A , t_0 and P_{orb} were allowed to vary so as

Table 1 Observations of 3U0352+30 on eight occasions between October 1972 and February 1977

Observation start	Observation length (d)	Mean time of Observation (d) from 1 Jan 1972	Pulse period (min)
28 Oct 72	3.4	304	13.9330 $\pm$ 0.0038
22 Dec 72	1.9	358	13.9302 $\pm$ 0.0049
2 Feb 74	5.3	766	13.9315 $\pm$ 0.0019
10 Jan 75	10.8	1,111	13.9212 $\pm$ 0.0010
11 Feb 76	7.5	1,506	13.9229 $\pm$ 0.0015
12 Sep 76	15.4	1,724	13.9188 $\pm$ 0.0011
8 Jan 77	18.8	1,845	13.92097 $\pm$ 0.00070
4 Feb 77	12.6	1,868	13.9196 $\pm$ 0.0011

to minimise χ^2 . The best fitting value of A is found to be 0.0030 ± 0.0008 min ($V \sin i = 65 \pm 17$ km s⁻¹), with the corresponding range of P_0 and P' being 13.9332 ± 0.0002 min and $-(2.72 \pm 0.07) \times 10^{-3}$ min yr⁻¹ respectively. The value of t_0 derived differed from that given by Hutchings *et al.* (JD 2442084) by 30 ± 30 d while, in the range 400–700 d, the optimum value of P_{orb} was 580 ± 30 d. For the purposes of assigning confidence intervals^{12,13} to the parameters derived, the uncertainties given in Table 1 were conservatively treated as 1σ gaussian errors. Figure 1 shows the data with the best fit linear trend taken out and folded about a 581-d period. The solid line represents the best fit sine curve whose amplitude, A , is equivalent to a circular orbital velocity of ~ 60 km s⁻¹.

Our data are thus consistent with a model in which the X-ray pulse period is modulated at the same period, phase and amplitude as the optical modulation found by Hutchings *et al.*¹¹ if an overall downward trend in pulse period is assumed. We consider this latter assumption to be justified since trends of this type are seen in other pulsating sources (for example

Table 2 Upper limits to the orbital inclination i for 3U0352+30

Period (h)	Predicted orbital velocity* (km s ⁻¹)	Upper limit to i from Copernicus data
11.2	763	15°
22.4	605	19°
44.8	480	25°

* Assuming a total mass of $22M_\odot$.

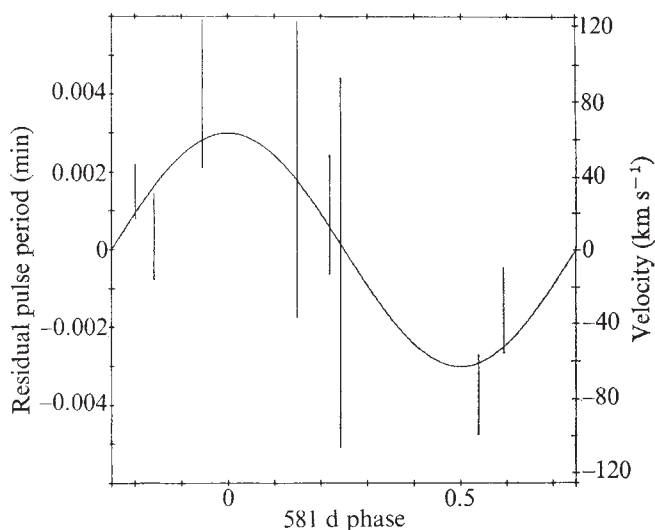


Fig. 1 The residual 13.9 min pulse period plotted as a function of the 581-d orbital phase. Phase zero represents X Per moving away from the observer at maximum velocity.

ref. 14). We recognise, however, that there are relatively few data, and it remains possible that we are observing (fortuitous) aperiodic fluctuations. More data are required over several 580-d cycles in order to bear out the effect. This will involve a substantial amount of effort because of the timescale of the phenomenon and the sensitivity required. Nevertheless we suggest that such an effort would be worthwhile since it could have important consequences for our understanding of the X Per system. In particular, if our present model of the data is correct we can make the following statements. (1) The secular decrease in period ($P/P \sim 10^{-4}$ yr⁻¹) suggests that 3U0352+30 is a spinning neutron star¹⁴ and not a white dwarf¹⁵. (2) The agreement in phase between the X-ray and optical modulations uniquely associates 3U0352+30 with X Per. (3) The amplitude of the X-ray modulation is consistent with the Balmer line shifts reported by Hutchings *et al.*¹¹. This is consistent with the premise of Hutchings *et al* that these lines give the true orbital velocity of the system (~ 60 km s⁻¹) and lends support to the idea that this is a triple system with both X Per and 3U0352+30 orbiting an unseen companion with a mass of several tens that of the Sun.

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On the orientation of the pulsar space velocity vector relative to the spin axis

A SIGNIFICANT parameter for pulsars for which proper motion data are available is the angle ϕ between the space velocity vector and the spin axis. There are eleven such pulsars (ref. 15 and personal communication from one of the authors). The angle between the spin axis projected on the plane of the sky and the proper motion vector has a value close to 0° for three of them, close to 90° for six and between 0° and 90° for the remaining two. We present here a hypothesis which would explain how the different orientations of the space velocity vector relative to the spin axis arise. We distinguish between three modes of formation of pulsars and show that these correspond to the three different sets of values of the angle.

The supernova explosion of a rotating magnetised star would result in the ejection of double jets along the spin axis¹, and only a part of the stored magnetic energy is sufficient to eject the supernova shell². Since in real stars the field is usually not at all symmetrical between the two poles, one would expect the jet ejection to be an asymmetric process resulting in the neutron star getting a kick along the spin axis. Asymmetric radiation, due to the dipole being off centre, from the resulting pulsar could give it an additional kick along the spin axis³. In other words, pulsars formed from the supernova explosions of single stars should have space velocities along the spin axis; pulsars with $\phi \approx 0^\circ$ (0531+21, 0833-45 and 2016+28) belong to this category.

For a supernova explosion to take place in a close binary system, the primary should have a mass $\gtrsim 15M_\odot$ (ref. 4). The primary evolves quickly, transferring more than two thirds of its mass to the companion before it explodes as a supernova, leaving behind a relativistic star: a neutron star or a black hole. Since the more evolved component is also the lighter (at the time of the explosion), mass ejection will not disrupt the binary⁵.

Two other mechanisms are available in principle for disrupting the binary. (1) The ejected supernova shell could impart a critical impulse to the unexploded companion making it leave the system⁶. It has been shown⁷, however, that this mechanism is not likely to be effective. (2) A highly asymmetric supernova explosion could impart a sufficient kick to the exploding star to push it into a hyperbolic orbit. To disrupt the binary in this manner, the impulse should be imparted in the direction of the orbital motion⁸. It is extremely unlikely that a kick normal to the orbital plane—assuming of course that the spin axis is perpendicular to the orbital plane—would disrupt the system. Highest observed space velocity for single pulsars is around 400 km s^{-1} (ref. 9). Velocities of this order are definitely unable to disrupt a binary system especially if the kick is normal to the orbital plane. So the first supernova explosion leaving behind a relativistic star core is unlikely to disrupt the binary. However, to account for the existence of runaway stars, it is necessary to disrupt some of the binaries at this stage. It should therefore be conceded that some supernova explosions do not leave behind any core¹⁰.

If a neutron star were formed in a massive binary it might tilt the orbital plane and impart a velocity of a few dozens of kilometres per second to the centre of the mass of the binary. It could also make the orbit eccentric but this would eventually become circular due to tidal action forcing the OB type main sequence star into synchronous rotation¹¹. The binary would manifest itself as an X-ray source as long as mass being accreted by the compact star from the OB type star (now a blue supergiant) is large enough to give a measurable X-ray flux¹², but not too large to choke the source¹³. Large mass loss would ensue when the primary evolves beyond the blue supergiant stage and overflows the Roche lobe. The X-ray source would be extinguished and matter start flowing out from the outer Lagrangian point. Enhanced accretion by the neutron star and mass loss from the system would spin up the neutron star¹⁴.

The explosion of the resulting helium star is likely to disrupt the system because it is more massive than the companion neutron star. (If the companion is a black hole, disruption probability would be smaller because then both the members of the system will have comparable masses). The disruption of the orbit would release two neutron stars. The old, reactivated neutron star would move in the orbital plane with its orbital velocity. So it would have a space velocity normal to the spin axis¹⁵. This is how pulsars with $\phi \approx 90^\circ$ (0823+26, 0834+06, 1133+16, 1237+25, 1929+10 and 2021+51) were liberated from binary orbits which were disrupted by the supernova explosions of the companion stars. The exploding star would get a kick along the spin axis¹ and so it will move with a velocity equal to the vector sum of the mutually perpendicular kick velocity and its orbital velocity. Pulsars with $0 < \phi < 90^\circ$ (0329+54 and 0950+08) are thus produced by the supernova explosions in and leading to the disruption of the binary systems.

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How did barium titanate particulates stick together in the nebula?

TANAKA *et al.*¹ have suggested that there must be barium-rich micro-components in the Allende meteorite. We have confirmed this prediction by discovery of barium-rich particulates (see ref. 2). Figure 1 shows diffuse distribution of Ba-rich particles as well as their clusters; each of apparent particles seems to be composed of finer particulates probably of submicron size. Tanaka and Okumura² showed that the Ba-rich spots are also high in content of titanium. Figure 2 shows the areal overlapping of the distribution of Ba with that of Ti. Relatively sparse dots for Ti are due to shorter scanning time than for Ba.

The results of electron microprobe studies² show the atomic ratio of Ba to Ti to be almost 1:1. Moreover, based on the data obtained by Tanaka and Okumura², contents of BaO and TiO₂ in most Ba-Ti-rich portion turn out to be 59 and 34% respectively; the pure chemical formula BaTiO₃ gives the theoretical values 66% BaO and 34% TiO₂. Thus the purity of micro-component as barium titanate is amazingly high. The small difference between actual and theoretical contents may be due either to experimental errors or to the presence of small amounts of other titanates and probably silicates. Palme and Wlotzka³ separated a particle (about $20 \mu\text{m}$) rich in refractory siderophile elements Os, W, Re, Ir, Mo, Ru and Pt from the Allende meteorite.

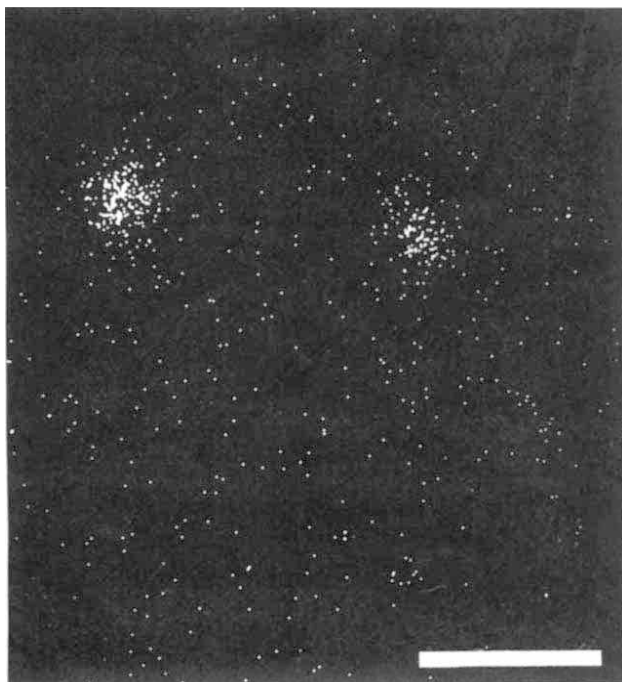
The Ba content of the Allende BaTiO₃ is therefore up to 1.7×10^5 times the corresponding chondritic abundance.



Fig. 1 Distribution of Ba microconcentrates in the matrix of Allende; scale bar indicates 10 μm .

This occurrence of barium titanate can be compared with that of calcium titanate (CaTiO_3), which occurs as perovskite in carbonaceous chondrite^{4,5}. According to Grossman⁶, the condensation temperature of perovskite is 1,647 K at 10^{-3} atm total pressure, implying that this mineral is one of the highest-temperature minerals to be condensed early in the solar nebula. It is important to know whether the condensation temperature of BaTiO_3 is higher or lower than that of CaTiO_3 and what the temperature difference is, but the condensation temperature of BaTiO_3 is not

Fig. 2 Distribution of Ti microconcentrates in the matrix of Allende; scale bar indicates 10 μm .



quantitatively known. It can be assumed from analogy, however, that this compound also has a high condensation temperature.

The Ba abundance⁷ in ordinary chondrites is between 3 and 4 p.p.m. The corresponding abundance⁸ of Sr is around 10–12 p.p.m., so the atomic abundance ratio of Sr to Ba is about 5. We might therefore expect the occurrence of SrTiO_3 in the same sample. Despite the careful X-ray scanning, however, it is significant that we have failed to detect the concentrates of Sr like those of Ba. Even apart from this observation, it is difficult to see how the barium titanate particulates could selectively stick to each other in the nebula. This is of even more interest when the low cosmic abundance of Ba is considered. Thus, is it conceivable that some special factor was operative in this selective sticking of BaTiO_3 particulates? Here we propose that the following unique properties of BaTiO_3 are associated directly with the formation of barium titanate particulates in these conditions.

Barium titanate has five crystalline modifications⁹: cubic, tetragonal, orthorhombic, trigonal, and hexagonal. Except for the hexagonal form which is stable above 1,733 K, other forms are well known for their highly dielectric and ferroelectric properties, while the dielectric constant is a function of temperature¹⁰. In particular, the tetragonal form which is stable between 393 and 278 K is known for being piezoelectric and ferroelectric. The Curie temperature is 393 K.

These physical properties imply that barium titanate fine particulates are liable uniquely to high dielectric polarisation in the presence of electric field and the electric charge is raised on their surfaces. It would be understandable that electrostatic situation like this is very favourable for selective attraction of BaTiO_3 fine particulates into larger particulate or aggregate. If BaTiO_3 particulates had still been floating in the nebula at the Curie temperature (393 K), the selective sticking of BaTiO_3 must have been promoted especially at this temperature (ref. 10). The electric field may have been prevailing due to paleosolar wind¹¹ or to friction of dust grains themselves, although other sources of electric field might be conceivable. It should be noted, however, that even without an electric field, spontaneous polarisation can arise for ferroelectric substance.

This reasoning in terms of electrical characteristics of BaTiO_3 seems to be parallel with the observation by Tanaka and Okumura²; the relatively large cluster of Ba–Ti-rich particles is found in matrix of meteorite rather than in inclusion composed seemingly of higher-temperature minerals. From the presence of magnetite in matrix of Allende, Larimer and Anders¹² estimated the upper limit of 400 K for accretion temperature of matrix. Consideration of the reaction of olivine with water vapour to form hydrous silicates led Anders¹³ to the reaction temperature range of 300–350 K. It should be noted that these numerical values possibly indicating the accretion temperatures for matrix are close to the Curie temperature of BaTiO_3 . (This does not conflict with our understanding that this compound was originally formed at high temperatures.)

As mentioned above, unlike Ba, the concentrates of Sr have not been detected in spite of the chemical similarity between both elements as alkaline earths. One of the plausible explanations of this fact is that the difference in ionic radius between Ca and Sr is smaller than between Ca and Ba, and Sr may therefore be more readily incorporated into calcium minerals. Alternatively, the relevant difference of Sr from Ba may be that the dielectric constant of SrTiO_3 is an order of magnitude lower than that of BaTiO_3 and, in addition, that the Curie temperature of the former is about 70 K, compared with about 400 K for the latter. These two possible explanations may not always be alternatives, but may be cooperative factors.

Discovery of pure ultra-fine particulates of BaTiO_3 opens new aspects in meteoritics. As maintained above, special

attention should be given to the unique electric properties of BaTiO₃. The very wide variation of Ba contents¹ must also be considered—the Allende meteorite does not represent the whole range of products which formed in a closed space box containing every element in proportion to cosmic abundances. Components of strange origin, even if solar, are certainly added in excess to the 'closed-space-box' products.

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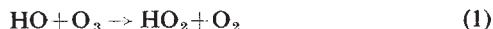
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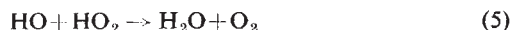
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Rates of reaction of HO₂ with HO and O studied by laser magnetic resonance

CONCERN about the depletion of stratospheric ozone by nitrogen oxides released by high flying aircraft¹ and by chlorine species from the decomposition of halocarbons^{2,3} has emphasised the need for laboratory data on the elementary reaction steps involved, particularly those which control the concentration of the hydroxyl radical (HO) in the stratosphere. HO has an important role in both ozone destruction mechanisms, since it combines with NO₂ to form the relatively inert HNO₃ molecule and converts the inactive HCl formed from Cl+CH₄ back into active Cl atoms. In the stratosphere the 'odd hydrogen' species H, HO, and HO₂ are formed by the reactions of O(¹D) with H₂O, H₂ and CH₄ and by the photolysis of H₂O, processes which are comparatively well understood. The odd hydrogen species are interconverted largely by reactions such as



and removed mainly by



The rate coefficients for reactions (1) to (3) are relatively well established^{4–6} but there is no previous experimental determination of k_4 and measurements of k_5 (which have not been based on direct and unequivocal measurements of the concentration of HO₂) have yielded values between 2×10^{-11} and 2×10^{-10} cm³ molecule⁻¹ s⁻¹ (refs 7–9). We report the first direct measurements of the rate coefficients k_4 and k_5 for which we have used the novel technique of laser magnetic resonance.

HO and HO₂ are detected in a fast flow system at total pressures of a few torr and 293 K by using a strong magnetic

field to bring Zeeman components of their $^2\Pi_{3/2}, \frac{5}{2} \leftarrow \frac{3}{2}$ and $4_{3,2} \leftarrow 5_{2,3}$ rotational transitions into resonance with the 118.6-μm line of a water vapour laser. The molecules are detected within the laser cavity and a thin, rotatable polyethylene Brewster window separates the laser discharge from the gas flow system (Fig. 1). The applied magnetic field is weakly modulated at 35 Hz and signals from a Golay cell, which monitors the laser output through a small hole in one mirror, are passed through a phase sensitive detector system and displayed in derivative form as in EPR. The lines show both Doppler and pressure broadening and, in the case of HO, can exhibit modulation broadening. The method of moments is therefore used to convert the differential traces into integrated absorptions from which the ratio of concentrations of HO₂ and HO can be calculated by using the matrix elements and g -factors for the transitions and the rotational partition functions^{10,11} and the dipole moments of the two species (S. D. Peyerimhoff, personal communication, refs 12 and 13 and G. Gillespie quoted in ref. 14).

Absolute sensitivities were established by generating known concentrations of HO using the rapid stoichiometric reaction



The reaction between HO and HO₂ has been investigated by studying three related chemical systems; in each system a stationary state in [HO₂] is established by reactions (7) and (5)



Here measurement of the absolute concentration of HO₂, together with the value of $k_7 = 7.9 \times 10^{-13}$ cm³ molecule⁻¹ s⁻¹ at 293 K (ref. 15) leads to a value for k_5 .

Possibly the simplest system is the reaction of H₂O₂ with HO which has previously been generated in reaction (6) conducted in the presence of a small excess of NO₂ over H atoms. Reaction (6), however, produces equal amounts of NO and HO; with the relatively long residence times used (~70 ms) the decay of HO on the walls ($k_w = 25 \pm 5$ s⁻¹) reduces the concentration of HO below that of NO at the point of H₂O₂ addition. Since reaction (8)



might cause errors in the analysis, other systems which avoid this complication were also investigated. In these, HO and HO₂ are generated by the reaction of H atoms with H₂O₂ and subsequently reactions (7), (5) and (9)



largely determine their concentrations. This reaction was allowed to proceed for about 30 ms and then sufficient NO₂ or O₃ was added to scavenge all the remaining H atoms with concomitant production of HO. Here the ratio of NO to HO is clearly unity if NO₂ is the scavenger and NO is absent when O₃ is used. The concentration of HO₂ present is then essentially governed only by reactions (7) and (5) and can thus be used to deduce k_5 . The central values of k_5 obtained from each type of experiment differed by less than the errors associated with the measurement of flow rates, signal strengths and so on, implying that nitrogen oxides do not interfere significantly in our conditions. The average value found for k_5 was $5.1 \pm 1.6 \times 10^{-11}$ cm³ molecule⁻¹ s⁻¹ at 293 K.

The reaction O+HO₂ was investigated in the reaction system

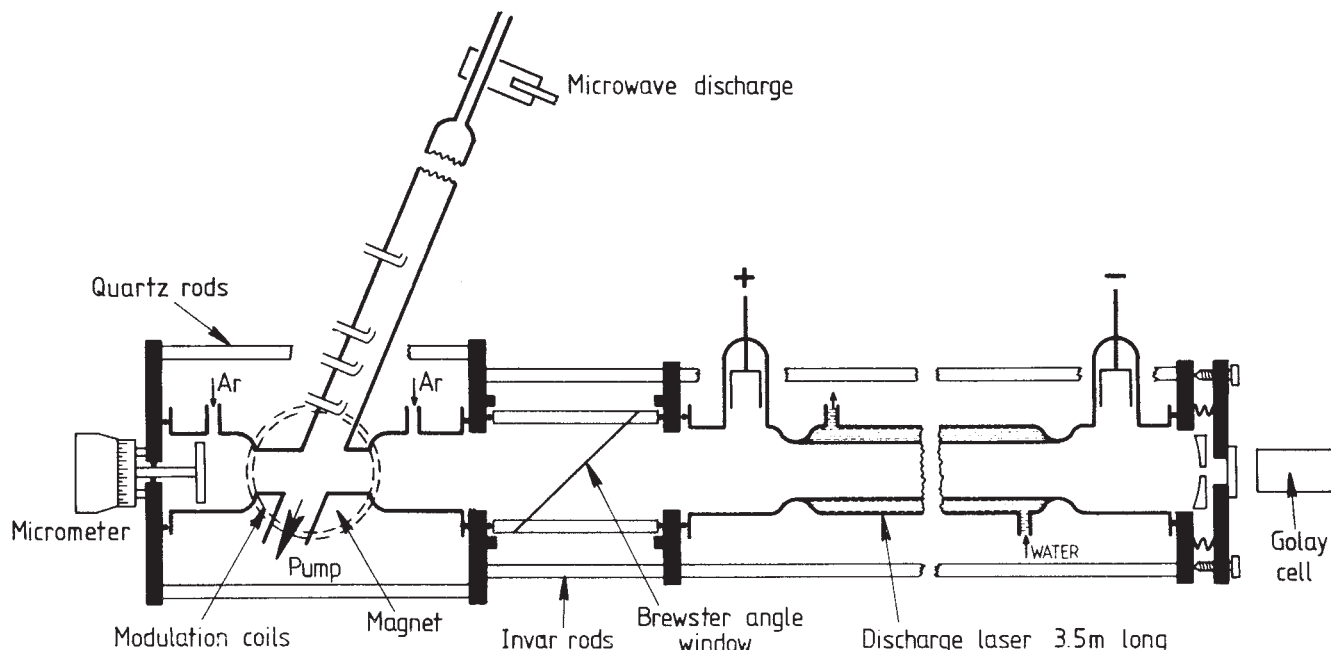


Fig. 1 Laser magnetic resonance spectrometer for kinetic studies.

O atoms plus H_2O_2 which is described by the following elementary reaction steps



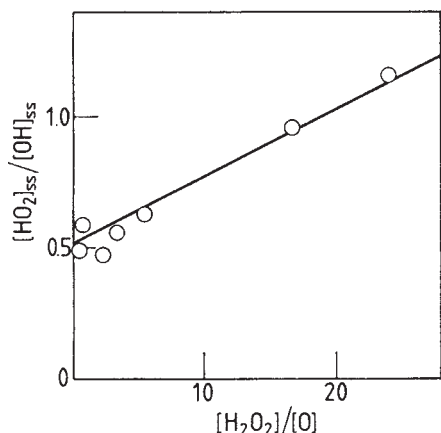
and the relative stationary state concentrations of HO_2 and HO are given by

$$\frac{[\text{HO}_2]}{[\text{HO}]} = \frac{k_3}{2k_4} + \frac{k_7}{k_4} \frac{[\text{H}_2\text{O}_2]}{[\text{O}]}$$

The ratio of the slope to intercept of a plot of this relationship (Fig. 2) is 4.7×10^{-2} which agrees very well with the literature value of the ratio $2k_7/k_3$ (refs 5,15) providing confirmation of the above mechanism. The average value of k_4 obtained from the slopes and intercepts of such plots is $k_4 = 3.53 \pm 1.0 \times 10^{-11} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ at 293 K.

The values of k_4 and k_5 are close to the expected pre-exponential factors for such reactions and the values should apply at stratospheric temperatures within the present accuracy of our measurements.

Fig. 2 Steady-state radical concentrations in $\text{O} + \text{H}_2\text{O}_2$ reaction.



A further interesting point is that the calculated concentrations of HO radicals in the middle and upper stratosphere are roughly proportional to $(k_4/k_5)^{1/2}$ and this ratio would not be affected by any inaccuracy in the calibration for HO_2 since both rate constants would be affected in a similar way.

Measurements of the rate coefficients of the $\text{H} + \text{HO}_2$ reactions and of the temperature coefficients of these processes are under way and the full details of these measurements will be described elsewhere.

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A new classification of metal catalysts in skeletal reactions of hydrocarbons

GENERAL knowledge about the factors governing the catalytic activity of various metals in different skeletal reactions of hydrocarbons is rather limited. Comparative studies are reported here on the depth of hydrogenolysis and the formation of saturated products of 3-methylpentane (3MP) and methylcyclopentane (MCP) for eleven different

metals. We have found that isomerisation, C_5 ring closure and opening activity of Rh, Pd, Ir and Pt can be correlated with their f.c.c. structure, and their atomic dimensions being in a narrow range. These four metals promote single hydrogenolysis whereas multiple fragmentation is the only reaction observed over Co, Ni, Cu, Ru, Ag, Re, Os. Hydrogenolysis activity and pattern are less characteristic for each group.

Various aspects of metal catalysed skeletal hydrocarbon reactions have been studied recently¹ to give some classification of various catalysts. For example, the depth of hydrogenolysis has been found higher over non-noble (Fe, Co, Ni) than over noble VIIIB group metals²⁻⁵; Re (and even Os) promote deep hydrogenolysis, too⁶. Cyclopentane C-C bonds have been reported to be similar to alkanes in their resistance to hydrogenolysis¹, although at least two mechanisms have been proposed for ring hydrogenolysis^{7,8}. Two mechanisms of skeletal isomerisation ('bond shift' and ' C_5 -cyclic') have been found over Pt (ref. 1); Pd, Rh, Ir, Ru have also been reported to isomerise *n*-heptane⁹. Hydrogenolysis has been described as a structure-sensitive reaction¹⁻³, and crystallite size effects may also influence the mechanisms of isomerisation¹.

We intended to compare various metals in conditions at which their catalytic activity is as close to each other as possible. Data in Table 1 are related to selected temperatures corresponding to nearly equal rates of 3MP hydrogenolysis. Hydrogenolysis activity is inversely proportional to the τ values. Crystallite size effects between 'powders' and 'blacks' are indicated by the fact that higher τ values are required in the former case to ensure identical activity per unit of surface. The depth of hydrogenolysis can be characterised by a fragmentation factor ζ . This is defined as the number of fragment molecules per molecule of hydrocarbon hydrogenolysed. This value is apparently independent of the structure of the initial hydrocarbon, the origin of the catalyst (that is, of its crystallite size) and the actual τ values at a given conversion level and given hydrogen pressure. C_6 product formation is characterised by the ratio of alkane isomers.

The metals studied seem to fall into two different groups. Group A contains four noble VIIIB metals—Rh, Pd, Ir, Pt—with ζ values near to two which means that single rupture of the molecule predominates. They promote isomerisation and C_5 -cyclisation of 3MP and produce very

high amounts of C_6 alkanes from MCP without altering the fragmentation yields and ζ values as compared with 3MP. On the other hand, the product spectrum over Group B metals including Co, Ni, Ru, Re, Os (and also Cu and Ag where τ was not reached) consists of fragments only except for Ru which produces traces of C_6 isomers. The ζ values between 3 and 6 indicate the multiple breakdown of the molecules. C_6 -alkanes represent a minor fraction of the product spectrum from MCP, their formation being apparently a part of the general scheme of molecular breakdown.

2MP/*n*H ratios show that alkane production from 3MP and MCP should be related processes; these ' C_5 -cyclic reactions' (including C_5 ring closure, opening and their combination: C_5 -cyclic isomerisation) are confined to group A metals. The higher rate, lower energy activation and different selectivity of ring opening indicates that it represents a particular type of C-C bond rupture distinct from either single or multiple alkane hydrogenolysis. Obviously, the difference can be attributed rather to the nature of the metal than to the structure of MCP¹⁰.

The pathways of cyclohexane dehydrogenation (through flatly or edgewise chemisorbed rings) have been correlated with the catalyst surface geometry, group A metals being the flatly adsorbing ones¹¹. These metals (and only they) have f.c.c. lattice with interatomic distances between 2.69 and 2.77 Å (ref. 12). With such a geometry, the spacing between interatomic holes on the closest packed (111) plane is appropriate (Rh: 1.55 Å Pt: 1.61 Å) for accommodating neighbouring carbon atoms of a hydrocarbon (C-C bonds between 1.522 and 1.546 Å (ref. 13)) over these sites. Such surface intermediates are suggested for single hydrogenolysis without disputing ideas about their nature or dissociated character¹⁻³. From Pt to Rh the ζ value remains almost constant (Table 1); with other surface geometry, the sharply increasing ζ values indicate that multiple hydrogenolysis should involve other surface species. We tentatively suggest that these are bonded to the top of metal atoms.

The concept of flatly chemisorbed C_5 -cyclic intermediate (suggested for C_5 -cyclisation over Pt (ref. 14)), can be extended for every C_5 -cyclic reaction over each group A metal. One active site may serve as a binding site (interacting preferably with the tertiary carbon atom), the other acting as a catalytic site for ring opening and/or closure.

Table 1 Comparison of 3-methylpentane and methylcyclopentane reactions over various metals

Metal	τ (K)	3 MP				MCP			
		< C_6 §	ζ	C_6 §¶	$\frac{2MP}{nH}$	< C_6 §	ζ	C_6 §**	$\frac{2MP}{nH}$
Co*	573	1107	4.99	0	—	3060	4.98	130	1.9
Ni*	543	463	3.13	0	—	380	3.11	120	2.8
Cu†	663	134	2.24	0	—	—	—	—	—
Ru*	513	545	3.76	0	—	1070	3.82	130	0.2
Ru†	543	544	3.71	3.3	~2	520	3.46	40	1.7
Rh*	513	591	1.86	34	—	75	2	655	1.6
Rh†	453	1100	1.80	0	—	550	1.78	6600	6.5
	498††	15450	2.17	330	5.6	11100	2.10	7460	6.5
Pd*	588	813	2.01	578	6.5	—	—	—	—
Pd†	648	610	2.08	1080	3.4	280	2.48	1630	2.9
Ag‡	723	22	2.55	0	—	—	—	—	—
Re*	633	805	3.45	0	—	690	3.37	120	0
Re†	573	782	3.35	0	—	1070	3.12	110	0
Os*	513	455	5.91	0	—	2070	5.97	90	0.5
Ir*	543	1000	1.92	410	6.4	1000	1.81	5900	5.7
Ir†	468	1060	1.76	430	—	1300	1.70	23600	32.4
Pt‡	663	278	2.09	980	4.9	—	—	—	—
Pt†	603	482	1.89	2420	5.7	320	2.14	9600	9.8

Pulse-microcatalytic system; strictly identical 3- μ l pulses introduced into 60-ml-min⁻¹ pure hydrogen carrier gas stream.

*Commercial powder. †Precipitated black. ‡Low surface sintered precipitated metal. §Expressed as 10¹⁵ C_6 molecule reacted per m² metal.

||Values lower than 2 may be due either to error in calculation or to actual methane deficit indicating irreversible adsorption. ¶Isomer C_6 alkanes (2MP, *n*H, eventually 2,2 DMB) and MCP. ** C_6 alkanes (2MP, 3MP, *n*H). ††Temperature for maximum isomerisation yield.

Such species also require definite surface geometry: with spacings that are too large (e.g. Ag) the chain ends cannot come together; with decreasing atomic diameter, the relative importance of hydrogenolysis as against isomerisation increases (cf. Table I), indicating that the attraction of next nearest neighbour sites from smaller and smaller distances gradually suppress C₅-cyclic reactions and promotes hydrogenolysis.

This concept is in agreement with the electronic inequality of tetrahedral (e_g) and octahedral (t_{2g}) sites¹⁵; it may also explain both the necessity of hydrogen for C₅-cyclic reactions¹⁴ and the selectivity (Fig. 1).

There are two possible explanations for c.p.h. Re and Os with proper atomic dimensions belonging to group B, in spite of the similarity of f.c.c. (111) and c.p.h. (0001) faces. The probability of occurrence of analogous crystallographic planes may not be equal¹²—higher hydrogenolysing activity on c.p.h. Ru and Os was explained this way¹⁶. Or the discrepancy may underline the role of sites where the geometry is favourable on f.c.c. metals only (e.g. steps or B₅ sites¹⁷) (Fig. 1).

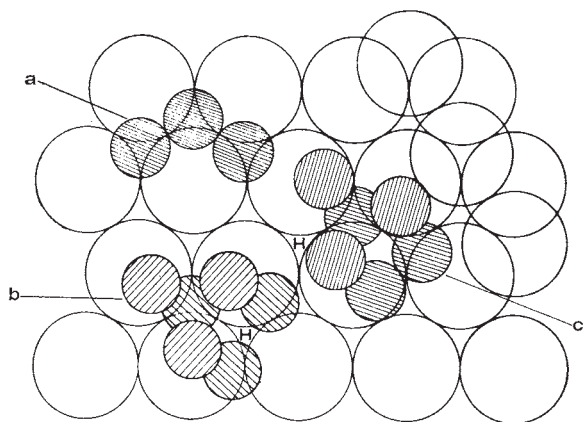


Fig. 1 Possible geometry of chemisorption over platinum (111) plane. The dimension of metal (larger circles) and carbon (smaller shaded circles) atoms is to scale. *a*, 1, 2, 3-chemisorption with participation of both e_g and t_{2g} sites leading to hydrogenolysis (1, 2- or 1,3-sorption is also possible); *b*, 3MP chemisorption over two neighbouring t_{2g} sites, with hydrogen atoms blocking more active e_g sites; *c*, one of the possible accommodations of MCP along a step [exposing (311) sites^{18,19}] ring opening over the B₅ site would give 2MP, over the neighbouring t_{2g} site 3MP; *n*-hexane formation is hindered.

We do not want to suggest that surface geometry is the only influence: the crystal parameters are outward manifestations of the collective interactions between individual atoms, but it should be pointed out that surface electronic inhomogeneity may create active sites the geometry of which may also be important in catalysis.

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Surface wind and sea waves in a hurricane field

THE hazards associated with a hurricane are extremely strong wind, violent seas and heavy rain. Over the seas, heavy rain does not pose as much problem as the other two. It is very rare to get detailed information of surface winds and waves in the hurricane field over the seas. The only set of documented data regarding surface wind and heights of waves is by Arakawa and Suda¹ describing the experience of main squadron of the Imperial Japanese Fleet as it inadvertently passed through the typhoon of September 1935. These simultaneous observations of weather and sea conditions are some of the best data on record. The hurricane reconnaissance over the Atlantic and Pacific could give the structure of winds in the hurricane field at 1000 ft or more above sea level, but no detailed observations about surface winds and waves are available.

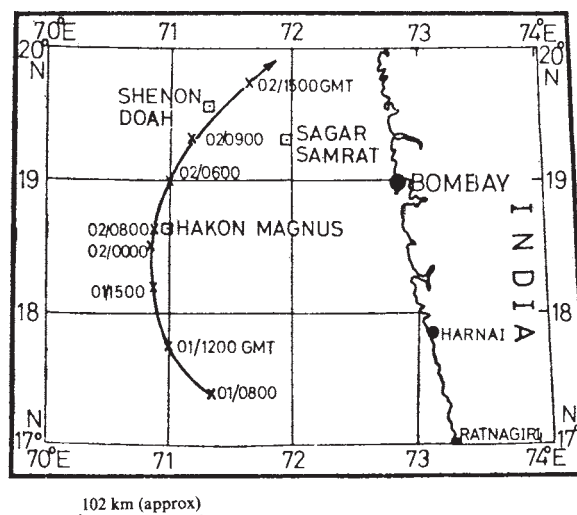


Fig. 1 Cyclone tracking by radar fixes of storm in Arabian Sea 30 May-3 June 1976, showing position of drilling rigs.

During the period 31 May-3 June, 1976, a cyclonic storm passed over Arabian Sea. On 2 June it reached hurricane stage and passed through Bombay High Area where offshore oil explorations are being conducted by the Indian Government. Fig. 1 gives the track of the storm with the positions of three jacked-up rigs: Haakon Magnus, Shenan Doah and Sagar Samrat. The storm centres referred to here are derived from the radar observations at the Area Cyclone Warning Centre, Bombay. It is a 10-cm radar with an effective range of 400 km. The rigs continuously recorded observations of wind and wave. The wind observations were made by standard wind instruments installed at the rigs and the wave measurements were taken with reference to the markings on the legs. These wave

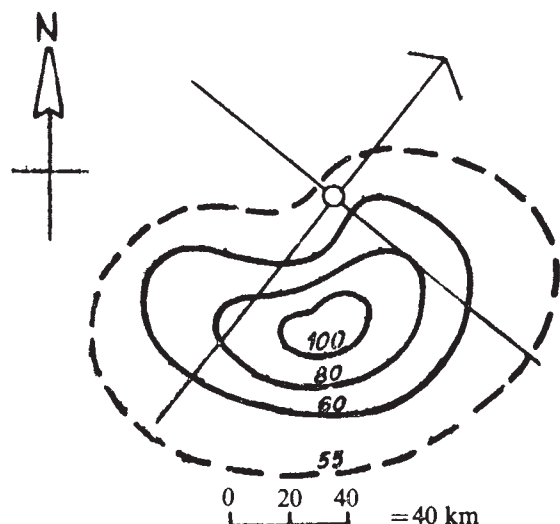


Fig. 2 Wind field over storm area, showing wind speed in knots. The arrow shows the direction in which the storm is moving.

observations are therefore taken to be more accurate than could be done on board a ship.

A composite wind speed chart was plotted throughout the day on 2 June. There was little variation with time; and the hurricane strength was maintained. The isotachs were drawn on this composite chart to determine the distribution of wind speed over different sides of the storm. This is shown in Fig. 2. It is seen that the strongest winds are found in the right rear quadrant and is very similar to the one found by Hughes² from aircraft reconnaissance of tropical storms over Pacific Ocean. Our observations differ from those of Arakawa who found the core of strong winds both in front and rear quadrants on the right side.

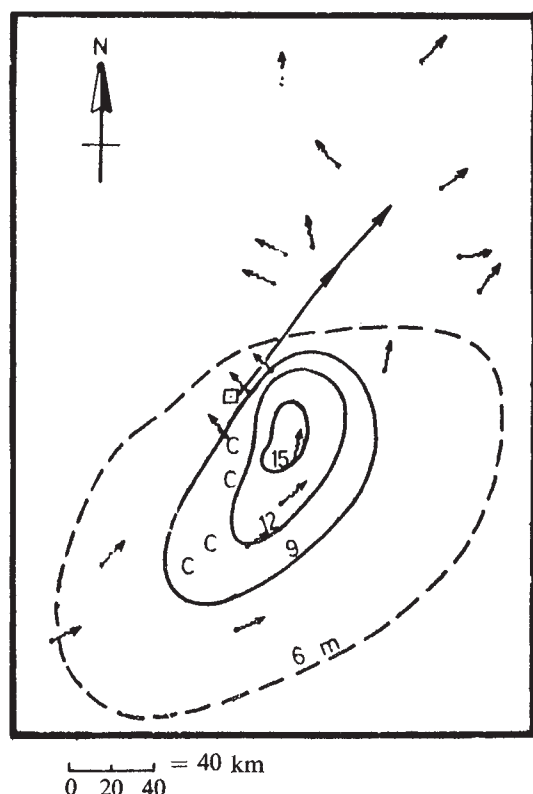


Fig. 3 Composite chart showing wave heights and directions. C indicates confused direction.

The composite chart for wind direction was also plotted (figure not presented). It was seen that the centre of the storm as determined by radar did not agree exactly with the convergence centre of wind pattern. The difference was found to be 20 km, the wind centre being to the right of radar centre.

The composite chart for wave heights and directions is shown in Fig. 3. The pattern is similar to one found by Arakawa for the sea in a typhoon, except that we found the highest waves both in the front and rear quadrants of the right side, whereas they found them in the right rear quadrant only. Wave directions generally agree with the theoretical model³. The sea was confused from the centre to the right rear quadrant.

The periods of waves varied between 6 and 12 s. The wave energy is given by $E = 41 H^2 T^2$ ft lb per foot of wave crest per wave length⁴. The maximum energies of waves encountered by the rigs were calculated (see Table 1).

Table 1 Maximum energies of waves encountered by rigs

Rig	Wave energy, E (10^6 J)
Sagar Samrat	1.72
Haakon Magnus	5.01
Shenan Doah	1.18

Energy values are given per metre of wave crests per wave length.

Assuming that the energy of wave is due to transfer of kinetic energy from air-water-surface interaction *in situ*, $E_{\max}$ is expected near the centre of the storm. This is confirmed by the value we get for Haakon Magnus.

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Imbricate thrust model of the Southern Uplands of Scotland

FAUNAL and structural evidence¹⁻⁵ suggest that a wide ocean was present between the Southern Uplands of Scotland and the English Lake District, and that during the late Ordovician and the Silurian the Southern Uplands were situated on the continental margin of North America, where the Iapetus Ocean was being subducted. Here we explain the stratigraphical and structural relationships observed in the Southern Uplands by a dynamic model involving sequential accretion of an imbricating sedimentary wedge on to the continental margin. This evolutionary pattern is analogous to those suggested for many modern convergent plate boundaries⁶⁻¹⁵.

The Southern Uplands has been divided stratigraphically into three units¹⁶ (Fig. 1). The Southern Belt is composed of Wenlock greywackes and shales, some of which contain graptolites¹⁷. The Central Belt consists of greywackes overlying condensed Moffat Shale successions of mid-Ordovician to Llandovery shales, ashes and cherts. In the

Northern Belt, which is entirely of Ordovician age, mafic volcanics, bedded cherts and, in some places, graptolitic shales underlie greywackes. The shales provided Lapworth with the data for his classic work on the use of graptolites as stratigraphic indicators; the seventeen zones of the Upper Ordovician and Lower Silurian have an average thickness of less than 5 m when present in this facies (see Fig. 2).

The general succession resembles many to be found in ocean basins today¹⁸, except that calcareous plankton were absent during the Lower Palaeozoic, and carbonaceous graptolitic shales occupy the position held by calcareous oozes in many modern oceanic sequences.

The rocks in the Southern Uplands have been interpreted as oceanic or trench deposits in the majority of previous plate tectonic reconstructions^{1,2,4,19}, but contrary views have been expressed based on the presence of high velocity rocks at depths of around 15 km below the Southern Uplands²⁰. On close inspection, however, the phase velocities recorded at the Eskdalemuir seismic array²¹ show considerable scatter, but there is a tendency for fast and slow-velocity source positions to be controlled by regional (ENE-WSW) strike. The slow velocities are obtained from positions in directions transverse to strike, and the fast velocities come from directions parallel to strike. It seems possible, therefore, that there is no sharp contact between low and high velocity rocks, and the higher velocities could be derived from metamorphosed Lower Palaeozoic sediments at depth.

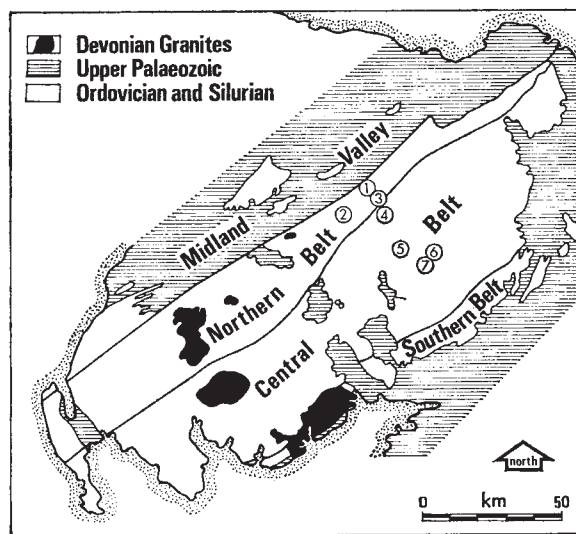


Fig. 1 Map of the Southern Uplands showing the location of the seven sequences in Fig. 2.

Our work in the Northern and Central Belts (unpublished) has substantiated previous observations of the importance of strike faults in the Southern Uplands²²⁻²⁸. Investigation (by J.K.L. and W.S.M.) of sections provided across the strike of the Northern Belt by the recent British Gas pipeline on a traverse to the west of the Clyde Valley has shown that, with only minor exceptions, beds dip at high angles and young predominantly to the north-west. Reverse faults are evident in zones of intense imbrication where the condensed basal basalt-chert-graptolitic mudstone units crop out, but they occur less frequently in the greywacke sections. In the Northern Belt, at least three major reverse faults must be inferred between observed zones of radically different stratigraphy (Fig. 2, sections 1-3). The Central and Southern Belt pipeline sections in Annandale (recorded by M.H.E.) showed steeply-dipping greywackes; though sometimes inverted, they were predominantly

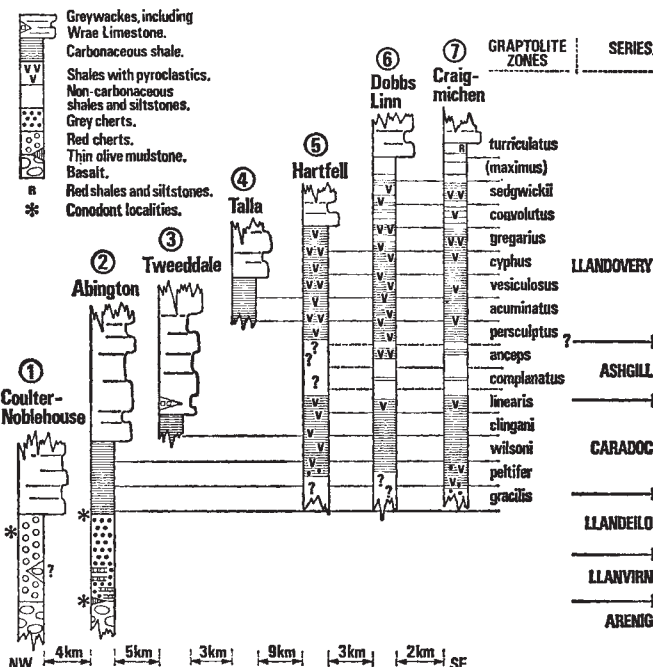


Fig. 2 Ordovician and Lower Silurian stratigraphy in the Southern Uplands: seven sections on a north-west/south-east traverse from the Southern Upland Fault in the Biggar area, Lanarkshire to the Moffat area, Dumfriesshire. The vertical scale reflects the ages of the beds. In the Central Belt (sections 4-7) the total thickness of the graptolitic shales and pyroclastic interbeds is up to about 80 m. Greywacke thicknesses in each fault block may be up to several kilometres. All sections are separated by faults (see Fig. 3). Beds with graptolites are linked by horizontal lines. Conodont localities from Lamont and Lindstrom³¹.

younging to the north-west. Lack of stratigraphic evidence precluded recognition of major fault-bounded sequences from the pipeline alone. A combination of this evidence with the well-established regional stratigraphy^{16,17,27,28}, however, shows significant differences in lithology and age of adjacent sequences (Fig. 2). In particular, it is estimated that the 26 graptolite zones of the Ordovician and Lower Silurian have a total duration of 80 Myr, an average of about 3 Myr per zone. The graptolite faunas thus show that the start of greywacke sedimentation differed by about 3-18 Myr between adjacent sections. We conclude that they were originally deposited far apart, and that they have subsequently been brought together tectonically.

Table 1 Deformation phases in the Moffat Shale of the Central Belt of the Southern Uplands

Style	σ_1	Phase
D ₁	335°	Thrusting southwards at a low angle to bedding, associated cleavage and isoclinal folding
D ₂	320°	Rotation of bedding and thrust planes, monocline formation
D ₃	340°	High-angled strike fault formation
D ₄		Wrench faulting
D ₅		Normal faulting

Structural analysis by M.H.E. from a number of Moffat Shale inliers suggests a complex evolution (Table 1). Initial low-angle southward thrusting, associated with some isoclinal folding was followed by rotation involving steepening, the formation of broad monoclines and steeply dipping strike faults. Such deformational sequences may be seen in certain inliers near Moffat such as at Dobb's Linn and Craigmichen, where beds are thrust over others of the same age but of considerable difference in lithology and thickness. Presumably sheet transportation must have been considerable to account for the juxtaposition of such dissimilar strata.

The model proposed here for the Southern Uplands (Fig. 3) is very similar to those suggested for many modern accretionary prisms. Along the Central American Trench⁶ there is imbricate thrusting and associated folding with progressive steepening, followed by uplift; this shows close analogies with the evolutionary development along such margins as those off Japan⁷, Washington and Oregon⁸, the Makran coast of Iran⁹, Peru¹⁰ and the Aleutians¹¹. Recently uplifted accretionary prism sequences are evident in Barbados^{11,12}, Alaska^{13,29} and the Mentawai Islands (Sumatra)¹⁴. In the Southern Uplands, Cockburnland, situated along what now constitutes much of the Northern Belt, became emergent by Upper Llandovery times and was a source of sediment for both the ocean to the south and the Midland Valley basin to the north²⁸. The younging of successive wedges towards the ocean coupled with overall younging of the sequence within each wedge towards the continent is diagnostic of accretionary prisms; this may be used as a test for their recognition in the geological record.

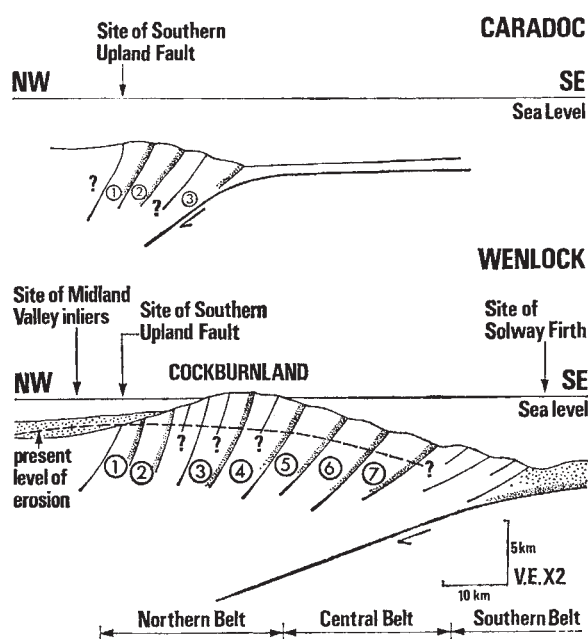


Fig. 3 The Southern Uplands accretionary prism in Caradoc and Wenlock times. Cockburnland was an emergent trench slope break in the Silurian. The numbers 1-7 refer to the sections in Fig. 2. Major faults separate each sequence; in addition, each sequence is affected by many minor faults. Only the known major faults are shown; there may be others, especially in some of the greywacke tracts (for example between sections 2 and 3). The shading at the base of the sections in the Northern Belt represents basalts and cherts, and in the Central Belt it represents shales and occasional cherts. The sediments in the Midland Valley and on the ocean crust to the south are also shaded. The remainder of the rocks are greywackes. The present level of erosion is arcuate to allow for post-Wenlock oversteepening of the prism.

All the evolutionary phases for the accretionary prism are assumed to be diachronous, occurring progressively later to the south. Uplift in the north occurred while accretion was still taking place on the oceanic margin of the Southern Uplands, and while sediments were still being laid down out on the ocean. The oncoming of the greywacke was progressively later to the south (Fig. 2). Similarly, the structural styles are diachronous across the prism; the D₁ to D₃ deformational phases represent only a systematic progression of style as a response to the same tectonic vectors.

With progressive accretion, the ocean margin of the prism migrated about 70 km southwards from a position

close to the Southern Upland Fault in the Llandeilo to the site of the Solway Firth in the Wenlock. Comparison with contemporary examples, such as the Aleutians, suggests that the angle of subduction decreased as the oceanic margin migrated southwards³⁰ (Fig. 3).

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Cretaceous and Tertiary dinoflagellates from Seymour Island, Antarctica

THE marine sediments on Seymour Island, north-eastern Antarctica Peninsula, have produced numerous plant, invertebrate, and vertebrate remains over the past 80 yr (ref. 1). These strata have been regarded as Campanian (late Cretaceous) and Miocene. Dinoflagellate assemblages, reported here for the first time, indicate that some of the Cretaceous beds may be older (Senonian) than previously suspected and that the supposed Miocene beds are instead late Eocene and early Oligocene in age. These Eocene-Oligocene strata are the only known marine sediments of that age exposed on the Antarctic Continent.

The first detailed mapping of Seymour Island began in 1973-1974 (ref. 2). The marine sediments on the island are traditionally divided into two main groups: the Snow Hill Island beds of early and medial Campanian (late Cretaceous) based on ammonites³ and the Seymour Island Series of early Miocene age based in part on fossil penguins^{4,5}. The material examined for dinoflagellates comes from four sections (Fig. 1) measured through these strata^{2,6}. The samples were collected principally by W. A. Bryant (US Geological Survey) and T. A. Trautman (Ohio State University) during the 1974-75 season.

The beds at the north-eastern end of the island adjacent to Cape Wiman that have been mapped as Cretaceous² may be

Palaeocene. A single dinoflagellate assemblage from 63 m above the base near the middle of section S-16 is dominated by an undescribed species and includes *Svalbardella australina*, *Hystrichosphaeridium tubiferum*, *Eisenackia*, and *Palambages*. The presence of these species indicates a Palaeocene correlation, although a possible very late Cretaceous age cannot be entirely discounted.

The northern half of the island, with the exception of the area adjacent to Cape Wiman, contains exposures of the supposed Miocene age Seymour Island Series. The 285-m section from the beach to the west edge of the meseta

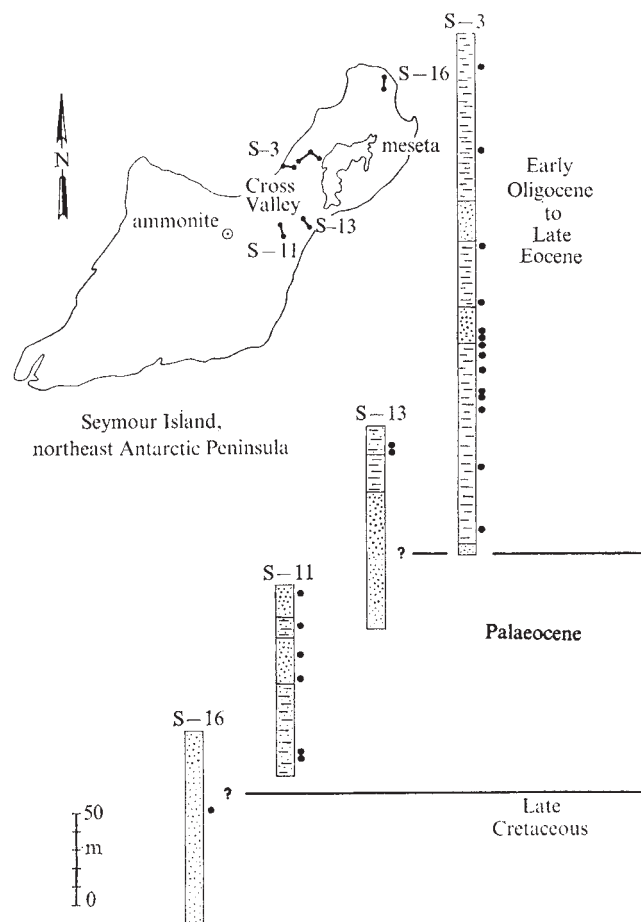


Fig. 1 Seymour Island ($64^{\circ}15'S$, $56^{\circ}45'W$) and generalised stratigraphic sections. Dots to right of columns represent samples from which dinoflagellates were recovered. The maximum length of Seymour is 20.5 km.

(section S-3) includes the oldest beds exposed in that part of the island. Sediments from the lower 84 m of the section contain a diverse dinoflagellate flora that includes *Aerospaeridium diktyoplokus*, *Deflandrea fuegiensis*, *Deflandrea macmurdoensis*, *Hystrichosphaeridium tubiferum*, and *Spinidinium aperturum*. Above 84 m occur *Aiora fenestrata*, *Cycloptella*, *Thalassiphora*, and *Tubiosphaera filosa*, in addition to the common forms *Deflandrea macmurdoensis* and *Spinidinium aperturum*. These species indicate a late Eocene–early Oligocene age for the Seymour Island Series. The dinoflagellate succession correlates in part with the flora from the Eocene Río Turbio Formation in southern Argentina^{7,8}, and similarities in species composition to the dinoflagellate assemblages from Deep Sea Drilling Project Site 274 of Leg 28 near the Antarctic coast are evident⁹. Several of the above species have been recovered from glacial erratics at McMurdo Sound^{10–13} and from mud

samples grabbed from the bottom of Ross Sea beyond the Ross Ice Shelf¹⁴. Lower Tertiary sediments that are the source of these erratics and dinoflagellates are not exposed in the McMurdo Sound region although beds which correlate with those on Seymour Island undoubtedly exist deeply buried beneath the continental ice sheet. The dinoflagellate evidence supports Simpson's¹⁵ conclusion that the fossil penguin materials from Seymour Island, presumably from the upper part of section S-3 or its equivalent², are no older than late Eocene and no younger than early Oligocene.

Eastern Cross Valley is thought to be the area from which the plant material that was examined for pollen by Cranwell¹⁶ was collected by the Swedish Antarctic Expedition of 1901–1903. The age of the pollen assemblage was initially reported as early Tertiary with recycled late Cretaceous forms¹⁶ and later re-interpreted as Maestrichtian–Palaeocene¹⁷. Section S-13 was measured in the Cross Valley fault zone. Only two samples (130 and 132 m above base) were examined for dinoflagellates. The lower one has a rich spore and pollen content but only one unidentified dinoflagellate; the upper sample is dominated by *Paralecniella indentata* which in Australia has a stratigraphic range from Campanian to Miocene¹⁸. Neither of the samples from S-13 provides conclusive information. It is likely, however, that the Cross Valley area contains a number of fault slivers which may include both late Cretaceous and early Tertiary sediments.

Section S-11 in the area mapped Cretaceous³ south of Cross Valley may be Palaeocene. Dinoflagellate assemblages from S-11 include *Chlamydophorella*, *Hystrichosphaeridium tubiferum*, *Palaeoperidinium*, *Spiniferites ramosa ramosa*, *Svalbardella australina*, *Tanyosphaeridium*, *Palambages* spp., and *Paralecniella indentata*; the ubiquitous *Deflandrea macmurdoensis* and *Spinidinium aperturum* of section S-3 are absent from S-11.

The southern half of Seymour has been dated by ammonite collections as late Campanian¹⁹ and early-medial Campanian³. Matrix from an unidentified ammonite collected by W. J. Zinsmeister (Ohio State University) from that part of the island (Fig. 1) contains the dinoflagellates *Cyclonephelium distinctum* and *Deflandrea cretacea*, indicating an early Senonian age for the ammonite, thus pre-dating the previous ammonite-based Campanian assignment.

Owing to the discovery of several previously undescribed faults in Cross Valley and to the results from the dinoflagellate investigation that differ from other palaeontological studies, final conclusions about the biostratigraphy and correlation of the southern part of Seymour Island will depend on further detailed mapping; at present the extreme southern part of Seymour Island has not been examined.

The abundant well preserved dinoflagellates of Seymour Island provide a new basis for unravelling what seems to be a more complex geology than anticipated. As the dinoflagellate and stratigraphic succession is studied it will become a standard for correlation of the other plant, invertebrate, and vertebrate remains with the better known and more accessible records elsewhere in the Southern Hemisphere.

In addition to abundant dinoflagellates, every sample examined except the one from S-16 contains a very rich assemblage of spores and pollen including several forms of *Nothofagus* and, from S-3, pollen of Gramineae. This is the first fossil record of grasses from Antarctica. Also, massula of the freshwater fern *Azolla* occur in S-11. These late Cretaceous–early Tertiary spores and pollen grains are unstudied but should provide a history of low latitude Antarctica vegetation during the Palaeocene–Oligocene period of climatic cooling before the formation of the present Antarctic Ice Sheet^{20,21}.

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Temporal characteristics of iconic memory

THE duration of visual persistence (iconic memory) is inversely related, up to a point, to the duration of the inducing stimulus. This suggests that iconic memory can more properly be identified with ongoing neural processes than with the decaying contents of a sensory store. Since the publication of Sperling's influential monograph¹ there has been general agreement that a relatively faithful representation of a visual display remains perceptually available for several hundred milliseconds after the distal stimulus has vanished. This led to the postulation of a short-term visual store, also called iconic memory² or sensory register³, with contents decaying rapidly after the termination of the inducing display. The terms visual persistence or icon are commonly used to refer to the perceptual availability of the decaying contents of the sensory store. Iconic memory is regarded as having temporal combinatorial properties which allow perceptual integration of two or more sequential displays even if separated by a brief temporal gap. Integration in iconic memory was convincingly demonstrated in a study where the subjects were required to identify which element in a briefly-displayed array of 16 alphabet characters had been singled out by a temporally trailing bar marker⁴. Temporal integration has also provided an explanation for some aspects of visual masking⁵ and for temporal integration of patterns whose parts were presented sequentially in time^{6,7}. Here I question the view that iconic memory is a store whose contents begin to decay when the inducing stimulus is terminated. I suggest that the duration of iconic persistence is linked to the duration of visual processes which begin at the onset of stimulation and continue for a given duration, whether or not the inducing stimulus is still on display. This view is based on the finding that the duration of visual persistence is negatively related to the duration of the inducing stimulus.

The experimental technique⁷ used a computer-driven oscilloscopic display (gridless Tektronix 602 equipped with ultrafast P15 phosphor) consisting of a square matrix of 25 dots arranged in five rows and columns. One of the 25 dots, chosen randomly on every trial, was not plotted. The subject, who sat in a dark room, initiated each display with a button press and then reported the matrix coordinates of the missing dot. The matrix was displayed in two successive portions separated by a temporal gap: first, an aggregate of

12 dots, chosen randomly from the matrix, was displayed for a period that varied between 10 and 200 ms, depending on the condition; next, a 10-ms temporal gap was allowed to elapse with no dots shown on the screen; and, finally the remaining 12 dots were displayed for 10 ms.

Figure 1 shows the results for two subjects. Almost identical results were obtained with other subjects and with casual observers in the laboratory. A marked impairment in performance is evident as the duration of the leading display exceeded about 100 ms. The phenomenal appearance of the display should be noted: at the shorter durations of the leading portion all dots in the matrix were perceived simultaneously with no evidence of a temporal gap. At the longer durations the display was seen clearly in two successive portions separated by a significant gap.

Since the experimental task was practically impossible unless the two portions of the display were actually seen as one, it seems likely that some sort of visual persistence was necessary for bridging the temporal gap. But could persistence be attributed to the rapidly decaying contents of an iconic store? Probably not. Bearing in mind that the temporal gap was of the same duration in every condition, and that a 'storage' theory assumes an icon whose strength does not start decaying until the termination of stimulation, it is difficult to see how the briefest (10 ms) display could induce iconic contents capable of bridging a given temporal gap while a much longer display could not.

More plausible inferences about visual persistence are, first, that its duration may be more closely timelocked to the onset than to the termination of the inducing stimulus, and second, that it may be more properly considered to be

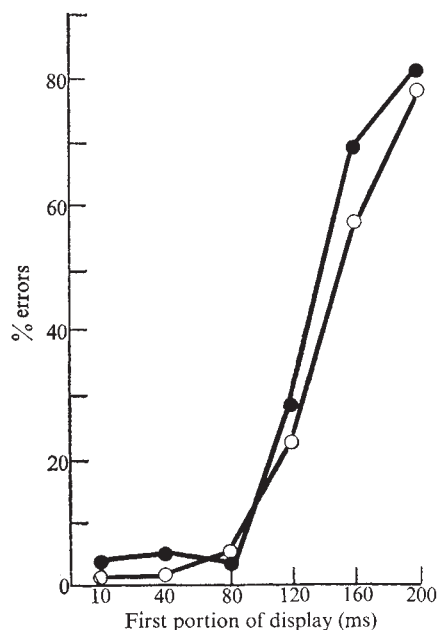


Fig. 1 Percentage of errors in identifying the location of a missing dot within a 5×5 -element dot matrix which was displayed on an oscilloscope in two portions of 12 dots each, separated by a 10-ms gap. The duration of the first portion of the display is shown on the abscissa; the second portion was always 10 ms. The matrix subtended approximately 6° of visual angle. The two portions of the display were equated for brightness through computer-controlled modulation of the oscilloscope's Z-axis. This allowed independent variation of the intensity of each dot in the display. The appropriate intensity levels for each duration of the leading display had been determined separately in a preliminary psychophysical task. Entirely similar results were obtained without brightness-compensation despite some degree of brightness-mismatch between the two portions of the display. Results are for two subjects—M.K. (●) and V.D.L. (○).

a product of ongoing neural processes of finite duration than the contents of a sensory store.

But before pursuing this line of reasoning, an alternative interpretation must be examined. It may be argued that impairment of performance at the longer durations of the leading display (Fig. 1) may have been due not to reduced visual persistence but to the triggering of some form of discontinuity-detection mechanism within the visual system which effectively segregated the two portions of the display one from the other^{6,8}. To examine this alternative, the display sequence was altered so that each of the 24 dots was displayed once only in random sequence at a regular inter-dot interval (with one exception as noted below). If the inter-dot interval is such that the total duration of plotting of the 24 dots exceeds about 120 ms, the matrix appears to have more than one missing element⁷. The observer is thus confronted with a choice among several apparently empty matrix locations which are indistinguishable from the truly empty location. Over 90% of the errors result from confusing the unplotted dot with dots plotted more than about 120 ms before the termination of the display and hence perceptually unavailable⁷.

The procedure of the second study was a modification of that of Hogben and Di Lollo⁷. The inter-dot interval was fixed at 10 ms corresponding to a total (24-dot) plotting duration of approximately 230 ms. In the control condition each of the 24 dots was plotted once only for 1.5 μ s. In the experimental condition each dot was plotted for 1.5 μ s except for the 12th dot which was plotted for 100 ms (Fig. 2). On the assumption that, up to a limit, the duration of visual persistence is inversely related to the duration of the inducing stimulus, it was expected that the sensory persistence of the 12th dot would be briefer in the experimental than in the control conditions. In turn, it was expected that the 12th dot would be erroneously identified as missing more frequently in the experimental than in the control conditions.

Two observers (the author and a student unaware of the purpose of the research) served in five experimental and five control sessions; each session consisted of 100 trials. The error distributions are shown in Fig. 3, separately for each

Fig. 2 Schematic representation of the plotting sequence. Each of the 24 dots was displayed for 1.5 μ s except for the 12th dot which was plotted for 100 ms. The 12th dot remained on view from the time of plotting of the second dot until 10 ms after the plotting of the 11th dot. Its brightness was indistinguishable from that of all other dots through the compensation procedure described in Fig. 1. The order of plotting was randomised on every trial so that every matrix location had an equal probability of being the 12th in the sequence.

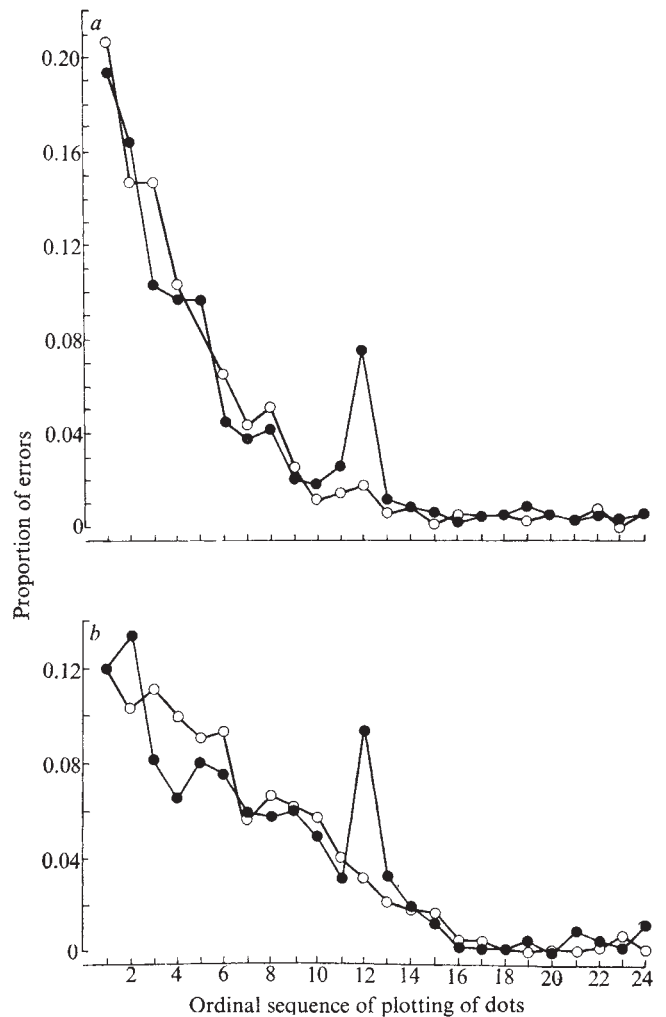
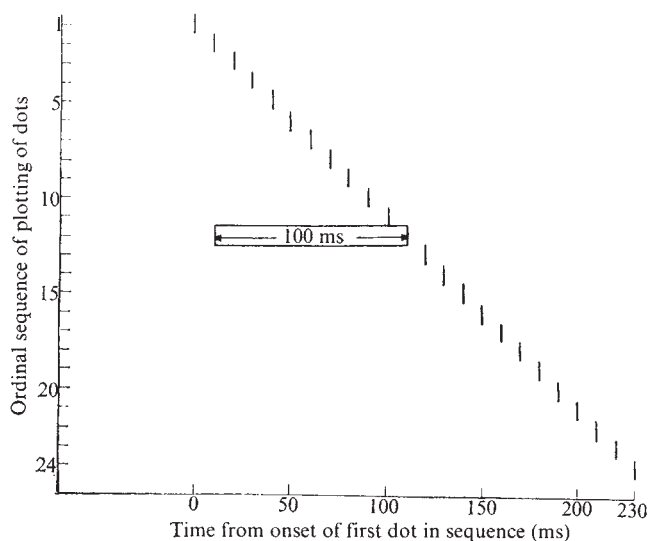


Fig. 3 Temporal distribution of errors in the results of the second experiment. Within each graph, the abscissa indicates the ordinal position of each dot within the plotting sequence, irrespective of spatial location; the ordinate shows the proportion of trials on which a dot plotted in the indicated ordinal position was incorrectly identified as missing. $\circ$, Control condition where every dot was plotted for 1.5 μ s; $\bullet$, experimental condition where all dots were plotted for 1.5 μ s except the 12th dot which was plotted for 100 ms. *a*, Subject L.D.C.; *b* V.D.L.

observer. Clearly, the probability of erroneously identifying the 12th dot in the sequence as missing was distinctly higher when its physical duration was long than when it was brief. Similar results were obtained with several other observers.

Unlike the first experiment, where perceptual segregation of the two portions of the display might have been possible, the continuous distribution of the dots over the entire plotting interval made segregation an all but irrelevant consideration in the second experiment. The results also deny the possibility that the termination of the 12th dot might have triggered some sort of discontinuity-detection mechanism within the visual system: had this been so, all dots plotted before the termination of the 12th dot would have become perceptually segregated from the remaining dots. On the contrary, the error distribution (Fig. 3) shows that the dots closely preceding or following the 12th dot in the display sequence were confused with the missing dot much less frequently than the 12th dot.

Contrary to most current thinking^{1-4,9} where iconic memory is said to begin at the termination of a display and to fade rapidly thereafter, the present findings suggest that

iconic memory (as distinct from a retinal after-image) begins at the onset of a display and continues only for a given duration (see also refs 10–13). Thus, iconic memory remains available after the termination of a display only if the duration of the inducing stimulus does not outstrip a given maximum duration from the onset of stimulation. This pattern of results strongly suggests that iconic memory may be more properly viewed as the product of neural processes of finite duration rather than as a sensory store whose contents begin to fade when stimulation terminates. The nature of the processes cannot yet be identified, but there is some support for the view that sensory persistence is produced by the activity of coding mechanisms at the level of feature extraction in visual information processing¹⁴. On this view, persistence would continue while the coding mechanisms are active and would cease at the termination of that processing phase, whether or not the inducing stimulus is still on display.

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Cuckoo among Lake Malawi cichlid fish

THE family Cichlidae consists of bony, perch-like fish which are found in many of the fresh waters of America, Africa and Asia. In Africa most of the almost 700 species occur in the Great Lakes. Approximately 300 species are known in Lake Malawi and 98% of them are endemic to it. Like cichlids from elsewhere, they have intricate but stereotyped territorial and courtship behaviour, followed by spawning and a period of parental care¹. Substrate spawners lay their eggs on the substratum or a suitable object and both parents guard the eggs and later the developing fry^{1,2}. In Lake Malawi the only known substrate spawner is *Tilapia rendalli* Dumeril, a non-endemic species. Evidence suggests that all other cichlids of Lake Malawi are maternal mouthbrooders. When eggs are laid they are picked up by the females and brooded in the mouth^{1,2}. When fry are ready to emerge the parent releases them at a suitable site and usually remains to guard her brood, recalling them into her mouth in the event of danger^{1,2}. After several days, fry no longer respond to their mother's recall signals and the association is terminated. Although cichlid parents usually guard their fry ferociously it seems that at least one species passes these parental duties on to other species of cichlid.

In May 1976, during diving off Likoma and Chisumulu Islands (Lake Malawi), maternal *Haplochromis polystigma* Regan were found caring for mixed broods: their own offspring, easily recognised because they have adult coloration and markings from the outset, and fry of another species differing slightly in size and markedly in coloration. *Haplo-*

chromis macrostoma Regan and *Serranochromis robustus* Regan were also found guarding mixed broods. The foreign fry appeared to belong in all cases to a single species, and differed sufficiently from the native fry to be readily distinguished. When prompted by divers, the guarding parents always retrieved their mixed broods. On occasion, they collected their own fry with greater facility than the foreign individuals, possibly due to a better response of their own progeny to recall signals.

As mixed broods were found on several occasions with each of the three parental species it is unlikely that foreign fry were included by chance. During the 10-d period at Likoma and Chisumulu Islands, *H. polystigma*, *H. macrostoma* and *S. robustus* parents were not found with pure broods.

Members of a mixed brood of *S. robustus* were caught and reared and the cuckoo was identified in January 1977 as *Haplochromis chrysonotus* Boulenger. *H. chrysonotus* is widespread in Lake Malawi and is a member of the Utaka group, which are plankton feeders³.

When the observations were made, shoals of *H. chrysonotus* brooding eggs or fry in their mouths were common in the surface waters. Surrogate parents were found beneath them on the rocks in 2–10 m depth. How the transfer of fry from *H. chrysonotus* to the foster parents takes place is unknown. The three species of foster parent are all active predators and it is conceivable that *H. chrysonotus* forms part of their diet.

It is easy to understand why *H. chrysonotus* progeny should accept foster parents because fry of maternal mouthbrooding cichlids show a very poor parental recognition response. They will follow and attempt to enter crude models which bear no resemblance to the parent in colour, shape, size or markings^{1,4}. Virtually all that is required to induce following is for the model to be moved in a manner which simulates calling behaviour.

It is not easy to understand why foster parents accept and care for fry markedly different from their own. Laboratory experiments^{5,6} with substrate-spawning cichlids have shown that broods could be swapped or mixed provided the progeny were very similar in age, size and behaviour. Dissimilarity of one or more of these criteria invariably resulted in the foster parent eating the foreign fry. Clearly the success of a cichlid cuckoo depends on accurate timing of introductions to the native brood and a similarity of behaviour. Furthermore, provided *H. chrysonotus* fry become intermingled in the foster parents' brood, then even if the parent were reluctant to accept them she would be unable to remove them. A predatory approach towards a mixed brood would evoke an avoidance reaction in all its members. In such circumstances foreign fry would be protected from the surrogate mother by shoaling with her own fry, and protected from other predators by their foster parent when they are taken into her mouth.

Cichlid fry reared with foster parents have been found to prefer the foster parental species to conspecific mates when placed in choice chambers at sexual maturity⁷. In natural conditions, however, *H. chrysonotus* and the three species of foster parent apparently breed true. It is therefore improbable that foster parents become imprinted on *H. chrysonotus* fry to such an extent as to impair subsequent recognition of conspecific rivals and mates.

Although *H. chrysonotus* brood their own eggs in their mouths, they are cuckoos in the sense that foster parents guard their young. This observation raises questions concerning the significance of the parent-brood relationship, the behavioural mechanism(s) of brood hybridisation, and the role of *H. chrysonotus* as a parent.

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Mode of action of insoluble monolayers on mosquito pupal respiration

THE emergence of resistant species and an increased awareness of the environmental problems associated with the use of insecticides has resulted in a search for alternative methods of mosquito control¹. One possibility is lecithin (phosphatidylcholine), which is lethal to the pupae of many species of mosquito and to some fourth stage larvae, when spread as a monomolecular layer at high surface pressure on the air–water interface^{2,3}. Other analogous water-insoluble surfactants, such as cetyl, stearyl and oleyl alcohol ethers of ethylene glycol are equally effective, and successful trials using several of these monolayers have been carried out on rice fields in Kenya⁴. These have not, however, thrown much light on the mechanism of the anoxic process. Observations by high-speed photography suggested that it is not simply a decrease in surface tension, with consequent wetting of the respiratory trumpet of the insect, but also involves a physical barrier to surface penetration (demonstrated in a 35-mm film available from A.I.M.). We report here the importance of the rheological nature of the monolayer, and show that those exhibiting ‘liquid’ properties at high concentrations form efficient barriers while ‘solid’ monolayers do not. We suggest that liquidity at high surface pressure enables (1) rapid repair of the monolayer after penetration or other disturbance; (2) rapid monolayer transfer to the penetrating trumpet during its displacement from the surface followed by ‘wetting’ and the formation of a bilayer-stabilised water ‘plug’, and (3) complete coverage of the water catchment surface in spite of, for example, projecting vegetation.

We applied 0.5 µg of several natural and synthetic lecithins and several semi-synthetic ethoxylated fatty alcohols as solutions in chloroform–methanol (2:1), to clean air–water interfaces within circular dishes of 5 cm internal diameter, each containing 40 pupae of *Aedes (Stegomyia) aegypti* in 8 cm of water at pH 5.8 and 27 °C. This amount of lipid is slightly in excess of that required to give complete coverage as a monolayer. We ensured that the pupae were submerged during the application and evaporation of the solvent. Differential surface

pressures (π) between the monolayer-covered water and clean water (also containing 40 pupae but treated with solvent only) were measured with a null-point electrobalance to each arm of which was attached a mica Wilhelmy plate about 1 cm wide. This gave the monolayer surface pressure as a function of time, with automatic compensation for solvent effects and pupal exudates (if any). The shear rheological properties of the monolayers were determined qualitatively by observation of movements of talc particles sprinkled on the film and gently subjected to an air jet. The surface pressure–molecular area curves obtained in separate experiments on an automated surface balance were used to observe the physical states of the lipid monolayers⁵.

Table 1 summarises the characteristics of the monolayers and their effects on pupae. The gel–liquid crystal transition temperature of lecithin bilayers (T_c) is the critical temperature for the transition from condensed to expanded monolayer⁵. When $T > T_c$, the monolayer is liquid-expanded at all film pressures up to the collapse point. π_s is the maximum film pressure observed when the monolayers were spread from solution and π_e , the equilibrium spreading pressure, is the pressure achieved by spreading from the pure solid⁶.

Table 1 shows that solid, condensed films do not cause death by anoxia, even at surface pressures close to 49 mN m⁻¹ while liquid-expanded films of similar chemical composition are effective at pressures down to about 40 mN m⁻¹. Previous work established a ‘threshold’ effectiveness at approximately 45 mN m⁻¹ (ref. 3). A simple explanation of the difference between solid and liquid films is that the solid film can readily be broken and partially collapsed by pupae; the hole is not immediately repaired because the lecithin cannot respread spontaneously because the value of π_e is zero^{5,6} and the solid ethoxylate film spreads to only 34 mN m⁻¹. On the other hand, similar breaks in liquid-expanded films are repaired quickly (because $\pi_e \approx 50$ mN m⁻¹), the rate depending on surface viscosity. Thus in the latter case, pupae are always faced with a complete and continuous monolayer at a very high surface pressure; this very high lateral pressure will increase the work required to create a hole in the film and tend to squeeze any trumpets out of the interface. We now consider the mechanism by which such films lead to anoxia of pupae.

In normal conditions, the pupal trumpet breaks surface readily without movement of the pupa other than vertical buoyant rise. Even in the presence of a liquid monolayer at $\pi > 45$ mN m⁻¹, pupae can usually gain access to the atmosphere at the first attempt but in subsequent attempts they struggle vigorously against the film, often concentrating the effort on one trumpet by attacking the surface sideways. When penetration is prevented, the air sac within the trumpet can be seen clearly like a stabilised foam bubble which does not coalesce with the atmosphere. Thus the interior of the trumpet can be represented as a bubble of air enclosed within a hydrophobic layer of material rising towards the surface (Fig. 1A). As the trumpet reaches the surface, water molecules must drain from between the two air phases, a^* and a , for these to coalesce, giving direct access of the respiratory system

Table 1 Characteristics of monolayers

Lecithin (or analogue)	Chain length and degree of unsaturation	T_c (°C)	Monolayer state at 27 °C	π_s (mN m ⁻¹ at 27 °C)	π_e	% Pupae dead after 12 h at 27 °C
Mainly						
Egg	C ₁₈ ^o _{18:1}	–15 to –5	Liquid-expanded	50	50	100
Total egg lipids	—	—	Liquid-expanded	48	48	100
Di-oleyl	C _{18:1}	–22	Liquid-expanded	46	46	100
Didecanoyl	C ₁₀ ^o	< 0	Liquid-expanded	45	—	100
Dimyristoyl	C ₁₄ ^o	23	Liquid-expanded	52	50	100
Dipalmitoyl	C ₁₆ ^o	41	Solid, condensed	49	0	0
Distearoyl	C ₁₈ ^o	58	Solid, condensed	49	0	0
Dibehenoyl	C ₂₂ ^o	75	Solid, condensed	37	0	0
Oleyl monoethoxylate	C _{18:1}	—	Liquid expanded	41	39	100
Cetyl/stearyl monoethoxylate	C ₁₆ ^o /C ₁₈ ^o	—	Solid, condensed	46	34	0

to the atmosphere. When the air-water interface in contact with phase a^* and the trumpet surface is devoid of surfactant molecules, drainage is rapid and rupture of the thin water film is easy (Fig. 1B); the hydrophobic trumpet is dewetted quickly because the receding contact angle θ_R of clean water is finite. This situation holds whenever the pupa penetrates a monolayer at less than the threshold surface pressure or for the first penetration of a monolayer at $\pi > 45 \text{ mN m}^{-1}$.

If a pupa detaches from a lecithin monolayer at $\pi > 45 \text{ mN m}^{-1}$ the bubble a^* becomes covered with a monolayer at high π and on subsequent contact with the surface the trumpet does not dewet quickly, because the air-water θ_R is zero (Fig. 1C and D). Brooks *et al.*⁷ have shown that θ_R for an aqueous surface covered with dipalmitoyl lecithin on low energy solids such as silicised glass decreases to zero when $\pi \approx 45 \text{ mN m}^{-1}$ (and tends towards zero when $\pi \approx 40 \text{ mN m}^{-1}$ in the case of octadecanol which is non-ionic and analogous to the ethoxylates); this wetting involves deposition of lecithin at the solid-vapour or solid-liquid interface. Because normal dewetting is thereby eliminated, a thin water film is maintained across the mouth of the trumpet and an air channel cannot be formed easily. In this situation, the thin film of water is analogous to that between air bubbles in a foam and it persists because the highly compressed lipid monolayers retard drainage of water from the film; ultimately a black film could be stabilised by interactions between the two monolayers⁸.

Although some species may penetrate a monolayer several times, progressive deposition of lipid and wetting will occur within the trumpet and it will become more and more difficult for the thickening plug of water (Fig. 1D) to drain in a reasonable time. Electron micrograph studies with *Culex pipiens fatigans* suggest that in such instances layers of surfactants are built up on the 'pedepodial' filaments within the trumpet, presumably destroying their function of resisting water invasion. That such deposits (Fig. 2) may be built-up multilayers of lipid is suggested by the observation that lipid multilayers can be deposited on a low energy surface such as PTFE (ref. 9).

Fig. 1 Normal dewetting of the hydrophobic pupal trumpet. In the absence of surfactant the thin film of water across the mouth of the trumpet is unstable because of the high free energies of the two surfaces. After rupture, the clean water recedes from the trumpet because the receding contact angle is finite (A and B). The effects of an insoluble monolayer with equilibrium spreading pressure $\pi_e > 45 \text{ mN m}^{-1}$ on the interaction of the pupal trumpet with the water surface are also shown. On the first approach (A) and (B) still apply because the air-water interface in contact with air inside the trumpet (a^*) is clean and the trumpet penetrates successfully. On subsequent approaches (C and D) this interface is covered with a monolayer of lecithin so that spontaneous dewetting of the trumpet no longer occurs, the receding angle now tending to zero, while the hydrated bilayer film is stabilised by minimal free energy interfaces.

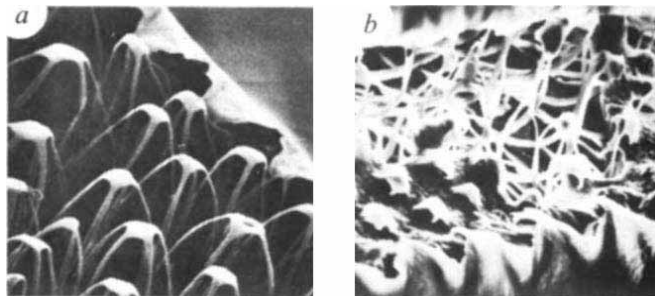
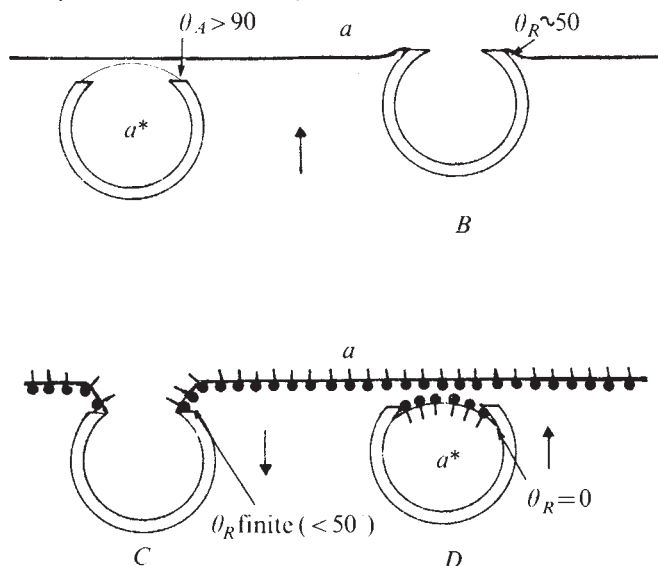


Fig. 2 a, An electron micrograph of the 'pedepodia' structures within a normal *Culex p. fatigans* pupal trumpet. b, A similar view of a specimen killed by lipid monolayer anoxia.

Laboratory and field studies indicate that many aquatic organisms ('non-target' in the context of mosquito control) are not killed by monolayers and we believe this to be due to the rather specific mechanism which we describe. Even the larvae of mosquito are not killed directly by monolayers, but are inhibited from surfacing until the dissolved oxygen content of the catchment falls below a critical value overnight¹⁰ when they are anoxiated.

When spread on tropical rice fields at about ten times the amount required for monolayer coverage, natural lipids and the semi-synthetic ethoxylates maintain their full surface pressure for about 1 and 3 d respectively. In both cases the monolayers, with their associated 'lenses' of reservoir material, effectively remove mosquitoes for periods of more than 10 d, after which further application would be required to give complete control.

These observations and our suggested mode of action indicate that natural lipid or other high-pressure monomolecular films afford a method of mosquito control which is more selective, more efficient and more ecologically acceptable than methods involving toxic chemicals. In addition, the physical nature of the mode of action which we have outlined may be less likely to encourage the selection of resistant mutants.

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Glutamine synthetase and control of nitrogen fixation in *Rhizobium*

Rhizobium, a bacterium which reduces dinitrogen to ammonia in symbiotic association with leguminous plants, 'fixes' nitrogen while living free¹⁻⁴. Unfortunately, free-living fixation seems to occur only at the end of exponential growth, and no condition is known in which bacterial growth is dependent on reduction of dinitrogen by nitro-

genase (EC 1.7.99.2). Therefore a molecular biological analysis must be indirect. In the unrelated *Klebsiella pneumoniae*, where fixation does occur in culture, nitrogenase expression is regulated by the enzyme glutamine synthetase (GS, EC 6.3.1.2). By analogy, we have isolated GS auxotrophs in *Rhizobium* and screened for nitrogenase activity. We chose *Rhizobium* cowpea 32H1 because it has the highest reported nitrogenase activity, both in culture and in nodules³. We found that a glutamine-dependent auxotroph of *Rhizobium* cowpea 32H1, deficient in GS activity, is deficient in nitrogenase activity as well. Therefore GS seems to be involved in the control of nitrogenase expression by *Rhizobium*.

In *K. pneumoniae*, mutants lacking GS activity (*gln*⁻) fail to induce measurable nitrogenase activity^{6,7}. Moreover, constitutive mutants (*Gln*^c phenotype), in which GS activity is no longer repressed by ammonia, are also constitutive for nitrogenase activity. On these grounds GS has been proposed as a positive regulator of nitrogenase.

In enteric bacteria GS has both catalytic and regulatory functions. Different GS assay procedures may yield different results. One assay (the 'transferase' reaction) measures ADP-dependent γ -glutamyl hydroxamate formation⁸. In this reaction the adenylylated form is still partially active. The adenylylated and deadenylylated forms of the enzyme GS, however, respond differently to changes in pH and Mg²⁺ concentration. Therefore quantification of GS levels is difficult unless the state of adenylation of GS is known⁹.

We have used the 'transferase' assay for rhizobial GS. Our finding that a GS auxotroph is deficient in nitrogenase is consistent with a similar regulatory role for GS in *Rhizobium*. We wish, however, to emphasise that little is known about rhizobial GS with regard to parameters such as adenylation and assimilation of ammonia at high and low concentrations.

GS transferase activity in wild type 32H1⁺ is shown in Table 1. Activity was five times higher in M + glutamate than in either M + glutamine or M + ammonia. This

Table 1 Glutamine synthetase activities of *Rhizobium* 32H1 strains

Strain	Growth medium	GS transferase activity (U per 10 ⁷ cells)
32H1 ⁺	M + glutamate	2.0
	M + glutamine	0.41
32H1 <i>gln</i> 5	M + glutamate	—
	M + glutamine	0.13
32H1 <i>gln</i> 5r 101 (<i>Gln</i> ⁺)	M + glutamate	0.47
	M + glutamine	0.39

To isolate glutamine auxotrophs, cells of 32H1⁺ were grown to 5×10^8 per ml in liquid cultures of YAP medium (0.1% yeast extract, 0.1% arabinose, 7.5mM phosphate, pH 6.3). Cells were incubated in citrate buffer, pH 5.5 containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.1 mg ml⁻¹) for 1 h at 30 °C and then washed with phosphate buffer, pH 6.3. Mutagenised cells were grown overnight in YAP and then resuspended in a minimal defined medium (M) containing: 0.1% arabinose, 0.8 mM MgSO₄, 0.05 mM CaCl₂, ferric citrate (10 μ g ml⁻¹) and 7.5 mM potassium phosphate, pH 6.3. Glutamate (0.1%) was added as nitrogen source and the culture was incubated at 30 °C for 24 h to deplete amino acid pools. At this time penicillin G (5 mg ml⁻¹) and lysozyme (0.1 mg ml⁻¹) were added and shaking was continued for a further 24 h. Surviving cells (approximately 0.1%) were treated with penicillinase and plated on YAP plus glutamine (0.1%). Among 1,500 colonies tested, three grew only when supplemented with glutamine. The reported experiments were conducted with one of these, *gln*5. GS was assayed by the glutamyl transferase reaction described in the text. 32H1⁺, 32H1 *gln*5 or independently isolated revertants growing exponentially at 30 °C in M + glutamate (0.1%) or M + glutamine (0.1%) were collected by addition of cetyltrimethylammonium bromide (0.1 mg ml⁻¹) and centrifuged in the cold. Cell pellets were washed once with 1% KCl, centrifuged and resuspended tenfold concentrated in the same. Assays were performed as before⁸, except that the pH was 7.00, where rhizobial GS transferase activity was a maximum. Twenty-five *Gln*⁺ revertants of *gln*5 assayed yielded the same non-derepressible phenotype when grown on M + glutamate as is shown by revertant 101.

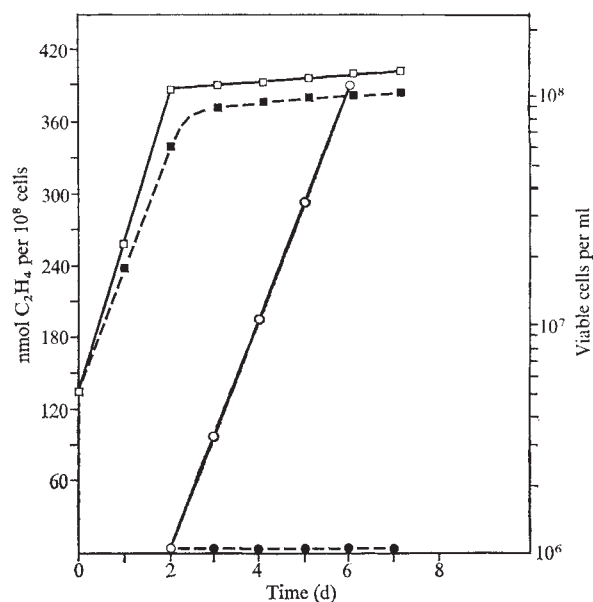


Fig. 1 Nitrogenase induction in liquid culture of 32H1 compared with 32H1 *gln*5. Nitrogenase was assayed by acetylene reduction in liquid culture¹⁰. 32H1⁺ and 32H1 *gln*5 were grown in YAP, centrifuged, washed with phosphate buffer twice and resuspended in M + glutamate (0.1%), glutamine (0.002%) and NaMoO₄ (10 μ g ml⁻¹). Cultures (3 ml) were injected into 30-ml capped serum bottles; the bottles were evacuated to 0.1 atm and the atmosphere was replaced with 95% argon–5% CO₂ four times in succession. O₂ (0.001 atm) and C₂H₂ (0.1 atm) were injected and cultures were rotated vigorously at 30 °C. Aliquots were withdrawn and plated on YAP + glutamine (0.1%) for cell counts: □, 32H1⁺; ■, 32H1 *gln*5; or assayed gas chromatographically for ethylene production: ○, 32H1⁺; ●, 32H1 *gln*5.

suggests that GS is sensitive to repression by ammonia in *Rhizobium*, as in *Klebsiella*; repression in M + glutamine might result from ammonia formed from hydrolysis of glutamine. (We will present a more complete study of repression by ammonia elsewhere.)

GS levels in the mutant *gln*5 can be measured only in repressing conditions (M + glutamine). Table 1 shows that *gln*5 had little or no GS activity, although a residual γ -glutamyl hydroxamate formed in the 'transferase' assay.

We cannot tell whether the lesion of *gln*5 is in the structural gene for GS or elsewhere. For what follows, the important point is that GS activity in *gln*5 is negligible.

Figure 1 shows nitrogenase activity in cultures of 32H1⁺ and 32H1 *gln*5. The mutant had no nitrogenase activity above background. Nitrogenase activity was assayed in liquid cultures¹⁰ by the reduction of acetylene to ethylene^{11,12}. Cells were inoculated at 10⁶ per ml. Before detection of nitrogenase activity colony counts reached 10⁸ per ml and then remained essentially unchanged for up to 10 d regardless of whether acetylene was present for nitrogenase quantification. If the vials were opened to the air, exponential growth resumed after some lag and continued to at least 5×10^8 cells per ml, showing that growth factors, particularly nitrogen, had not been depleted. Glutamine was present at an unusually low level (0.002%) because higher levels inhibit nitrogenase induction in 32H1⁺.

To study the effect of the *gln*5 allele on symbiotic nitrogen fixation, 32H1⁺, 32H1 *gln*5 and *Gln*⁺ revertants of *gln*5 were allowed to nodulate the tropical legume *Macropitium atropurpureum*. Seedlings nodulated with 32H1⁺ showed high levels of nitrogenase activity whereas there was no detectable activity either in plants nodulated with *gln*5 or in uninoculated, un-nodulated plants (Fig. 2). *gln*5-inoculated seedlings grown for a further 14 d as a precaution against slow maturation still had no nitrogenase activity.

Seedlings with effective nodules were green and healthy, whereas ineffectively nodulated seedlings began to yellow

Table 2 Nitrogenase activities and phenotypes of viable *Rhizobium* of *M. atropurpureum* nodules

Strain inoculum	Viable cells of <i>Rhizobium</i> * per nodule	Phenotypes*		Nitrogenase activity†
		Gln ⁺ (%)	Gln ⁻ (%)	
32H1 ⁺	7.0×10^6	100	—	204
32H1 <i>gln5</i>	5.8×10^6	83	17	<1
r101 (Gln ⁺)	6.1×10^6	100	—	112
r102	3.6×10^6	100	—	<1
r111	8.0×10^6	100	—	<1
r113	3.2×10^6	100	—	<1
r114	4.1×10^6	100	—	86
r16L	5.3×10^6	100	—	<1

Seedlings were grown and nodulated with the given strains of *Rhizobium* 32H1⁺, 32H1 *gln5*, and Gln⁺ revertants as described in Fig. 2. Assays for nitrogenase were conducted as described in Fig. 2. Nodules were excised, immersed momentarily in ethanol, then in 3% H₂O₂ for 5 min, crushed in 1.0 ml of phosphate buffer, pH 6.3 and plated on YAP and YAP + glutamine (0.1%) for viable cells of *Rhizobium*.

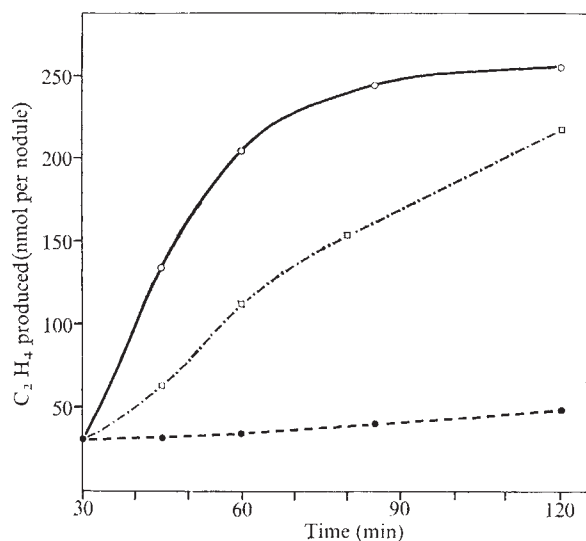
*Mean number of six nodules.

†Net activity in nmol of ethylene produced per h per nodule minus that produced by uninoculated, un-nodulated seedlings.

due to acute nitrogen starvation. Both ineffective and effective nodules were then crushed and plated for counts of viable *Rhizobium* with both Gln⁻ and Gln⁺ phenotypes. We were surprised that nodules resulting from *gln5* inoculation yielded viable *Rhizobium* in a mixture of both phenotypes, with Gln⁺ predominating (Table 2).

Nodulation with both 32H1⁺ and 32H1 *gln5* was repeated with various amounts of glutamine (0.01–1.0%) added to the seedling media. No nodules were seen on plants inoculated with either strain in the presence of added glutamine. But between one and five nodules per plant eventually developed during the next 14–28 d. As expected, nodules produced from *gln5* inocula had no nitrogenase activity. Again, aseptic crushing and plating for viable *Rhizobium* revealed populations with mixed Gln⁻ and revertant Gln⁺ phenotypes. Therefore *gln5* might itself nodulate and the Gln⁺ colonies represent simply accumulated revertants. Alternatively, *gln5* might be deficient at some stage of nodulation at which reversion to prototrophy is required. In this case *gln5* would only passively inhabit nodules formed by revertants.

Fig. 2 Nitrogenase activity of *M. atropurpureum* nodules. Seeds were surface sterilised in hypochlorite solution (2.5%) for 30 min, sprouted and placed in 70 × 25-mm tubes with filter paper supports. Seedlings were grown in nitrogen-free liquid medium¹⁷ inoculated with 10⁶ exponentially growing 32H1⁺ (○), 32H1 *gln5* (●) or 32H1 Gln⁺ revertant r101 (□) 7 d after germination. After 28 d of growth at 29 °C with a constant 14-h photoperiod, well-nodulated seedlings were capped and injected with C₂H₂ (0.1 atm). Aliquots were withdrawn periodically and assayed for ethylene production by gas chromatography^{11,12}. No ethylene production was observed in uninoculated, un-nodulated seedlings or *gln5*-nodulated seedlings. Activities are given per effective nodule, which was determined by subsequently excising individual nodules and assaying for ethylene production.



In culture, 32H1 *gln5* reverts to prototrophy at a rather high frequency of 1×10^{-5} per cell per generation. Six such independently isolated Gln⁺ revertants of *gln5* were tested for nodulation and activity (Table 2). Two gave nodules that reduced acetylene at rates similar to 32H1⁺ nodules. The remaining four gave nodules that looked healthy but had no detectable nitrogenase activity. This suggests that there are two classes of Gln⁺ revertants: those that regain both catalytic and regulatory activities, and those that regain catalytic activity only. (We have no selection for revertants that regain regulatory activity only.)

These results indicate that GS plays a role in the regulation of nitrogenase activity in both free-living *Rhizobium* and bacteroids, although the mechanism is still unclear. Kondorosi *et al.*¹³ have isolated a glutamine-requiring derivative of the 'fast-growing' species *R. meliloti* 41 his⁻ from among the revertants able to grow on D-histidinol. This glutamine auxotroph yields nodules on *Medicago sativa* which do not have nitrogenase activity, a finding similar to our own. The results of Brown and Dilworth¹⁴ with bacteroid preparations suggest that glutamine synthetase–glutamate synthase-directed ammonia assimilation does not occur in the bacteroid, but rather in the associated plant cell where the same two plant enzymes are present at high levels. These plant extracts also had NAD- or NADP-linked glutamate dehydrogenase activities. Bergerson and Turner¹⁵ showed that all ¹⁵N₂ reduced by *R. japonicum* bacteroids appeared rapidly as ¹⁵NH₄ in supernatant fractions, supporting the involvement of plant ammonium assimilatory enzymes in fixation. O'Gara and Shanmugam confirmed that 94% of the ammonium ion produced by nitrogen fixation in free-living *R. japonicum* is exported as ammonium ion¹⁶.

In both free-living and bacteroid states, rhizobial GS seems to be present during fixation at low levels, insufficient to utilise all the ammonium ion produced by nitrogenase. Because bacteroids are inviable differentiated forms, rhizobial GS might be involved in shutting off the assimilation of bacteroid ammonia as part of the overall shift in nodule metabolism from bacterial to plant cells.

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Nitroso group exchange as a way of activation of nitrosamines by bacteria

MUTAGENIC compounds and most carcinogens are thought to interact either directly or through metabolic activation processes with DNA to alter and damage the double helical structure. Bacteria defective in some aspect of DNA repair or recombination are in general much more sensitive to mutagenic and carcinogenic agents than the parent strains. Several bacterial test systems^{1,2} have been developed to assay for carcinogenic activity based on the premise that most carcinogens are mutagens in the proper conditions. One of the most widely used bacterial screening systems has been developed by Ames *et al.*¹ based on mutational reversion to prototrophy of a sensitive strain of *Salmonella typhimurium*. We have developed a screening system using *rec* *Escherichia coli* based on the lethal nature of unrepaired DNA³⁻⁵.

During the development of this screening system we discovered that our test bacteria, a *recB⁻ recC⁻* (*recBC*) strain obtained from A. J. Clark, Berkeley, California, was very sensitive to a mixture of 2-acetyl aminofluorene (AAF) and dimethylnitrosamine (DMN), neither of which alone

Fig. 1 Alkaline sucrose gradient analysis of *E. coli* AB1157. Cultures in exponential growth were labelled with ³H-thymidine, 3 μ Ci ml⁻¹ (specific activity 50 Ci mmol⁻¹) at 37 °C for 1 h in the presence of 2-deoxyadenosine, 250 μ g ml⁻¹. Bacteria were then centrifuged, resuspended in 0.1 M phosphate buffer (pH 7.0) and treated with DMN and/or AAF for 20 min. They were then washed twice to remove the carcinogens and resuspended in 0.1 M phosphate buffer (pH 7.0). Following this, 50 μ l (~10⁶ cells) were layered gently on 0.15 ml lysing solution (0.15 N NaOH, 0.40 M NaCl, 0.01 M Na-EDTA and 0.3% sarcosyl) placed on top of 4.3 ml alkaline sucrose gradient (5-20% sucrose, 0.9 N NaOH, 0.1 M NaCl and 0.001 M Na-EDTA) and a shelf of 0.5 ml 70% sucrose. The gradients were allowed to stand for 1.5 h or 90 min at room temperature to ensure complete lysis of cells and then centrifuged for 1 h at 40,000 r.p.m. in a SW65 rotor in a Beckman L-65 ultracentrifuge. Eight drop fractions were collected from each gradient and sample counted in a Triton 100-toluene fluor. ●, AAF 25 μ g ml⁻¹; □, DMN 2.5 $\times 10^{-1}$ μ g ml⁻¹; ○, AAF 25 μ g ml⁻¹ plus DMN 2.5 $\times 10^{-1}$ μ g ml⁻¹.

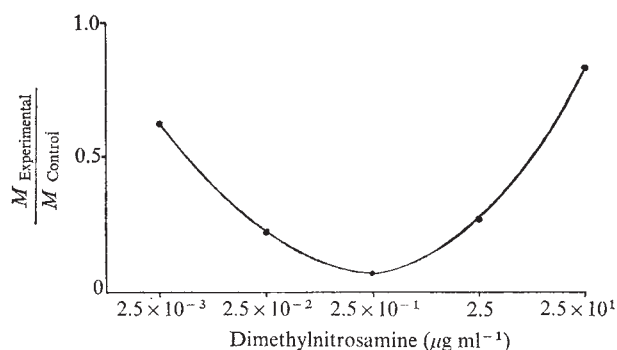
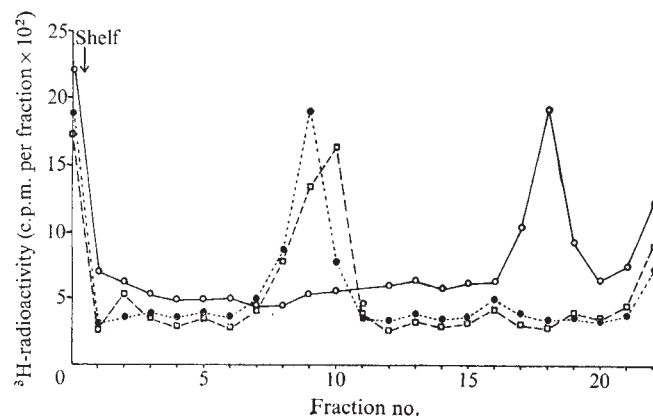


Fig. 2 Alkali sensitivity of bacterial DNA as a result of exposure of bacteria to AAF plus DMN. Using the procedures given in Fig. 1, a number of alkaline sucrose gradients were analysed in which the AAF concentration was kept constant at 25 μ g ml⁻¹ and the DMN concentration was varied from 2.5 $\times 10^{-3}$ to 2.5 $\times 10^1$ μ g ml⁻¹. The ratio of the molecular weight of the DNA of bacteria treated with AAF plus DMN (*M* experimental) to the molecular weight of the untreated control (*M* control) or to the molecular weight of the bacteria treated with AAF or DMN alone was obtained from the relationship:

$$\frac{\text{Distance}_1}{\text{Distance}_2} = \left\{ \frac{M_1}{M_2} \right\}^{0.4}$$

as developed by Studier⁶.

had any effect on the growth of JC5519 (*recBC*). In the presence of 25 μ g AAF, the assay could detect as little as 2 $\times 10^{-2}$ μ g DMN without the requirement for metabolic activation which is normally required for DMN. Was this synergistic effect acting in a 'classical' manner on the genome of the bacteria?

Cells of strain AB1157 (the parental strain isogenic with JC5519), whose DNA had been radioactively labelled, were exposed to AAF, DMN and a mixture of AAF and DMN, then lysed on top of an alkaline sucrose gradient and sedimented⁶. Figure 1 presents the results of such gradients which show quite clearly that the combination of AAF and DMN produce alkali-labile lesions (single-strand breaks) in DNA. On the other hand, AAF and DMN alone have no effect on the DNA of the bacteria as observed in alkaline gradients with control bacteria. If the AAF concentration is kept constant and one varies the DMN concentration, the fragment size as observed in alkaline sucrose gradients first decreases as the DMN concentration increases, reaches a minimum size and then increases as shown in Fig. 2. At high levels of DMN the combination of AAF and DMN has no effect on the bacterial DNA probably due to cytotoxic effects.

To explore the hypothesis that a bacterial enzyme was involved in this interaction between AAF and DMN, we incubated bacterial extracts from both AB1157 (parent strain) and JC5519 (*recBC*) with AAF and DMN, and tested them with the Ames tester systems. AAF and DMN without microsomal activation have a negligible effect in producing revertants in the *his* *S. typhimurium* tester strain; however, in the presence of sonicated bacterial extracts from AB1157 or JC5519, the combination of AAF and DMN became mutagenic in the standard Ames assay (Table 1). As a next step, after incubation of AAF and DMN with a sonicated cell-free bacterial preparation, we extracted the reaction mixture with methylene chloride, evaporated the methylene chloride fraction to dryness, and dissolved the residue in DMSO. This DMSO extract was also active in producing revertants in *S. typhimurium*. These results are presented in Table 1. In Fig. 3 we have plotted the results from a number of assays with the Ames tester strain; the AAF concentration was kept constant and the DMN concentration varied in the presence of JC5519 bacterial extract. The number of revertants rise with DMN

Table 1 Mutagenic activity of cell-free preparations of *E. coli*, AAF and DMN, using *S. typhimurium* TA98

Compounds of mixture	Mutation ratio TA98
1 AAF+DMN+JC5519 prep	2.8
2 AAF+DMN	1.0
3 DMN+JC5519 prep	1.0
4 AAF+JC5519 prep	1.0
5 AAF+DMN+JC5519 prep (heated)*	1.0
6 AAF+DMN+JC5519 Prep (pronase treatment)†	1.0
7 AAF+DMN+AB1157 prep	1.7
8 AAF+DMN+CH ₂ Cl ₂ extract‡ of JC5519	2.3
9 AAF+benzylphenyl nitrosamine+JC5519 prep	2.5
10 AAF+nitroso-pyrrolidine+JC5519 prep	2.4

E. coli JC5519 were grown overnight on nutrient broth, and collected by centrifugation at 4,000g for 15 min. The bacteria were resuspended and washed twice in phosphate buffer (0.1 M, pH 7.0) and finally suspended in 50 ml to give about 10⁸ cells ml⁻¹. The cell suspension was sonicated at full power in a Bionsonik sonicator for 5 min, and cooled in an ice bath. The sonicated preparation was centrifuged three times at 4,000g for 15 min to remove intact bacteria. The final supernatant was used for subsequent experiments. The protein concentration of the preparation was routinely 2.0 mg ml⁻¹ by Folin-Lowry.

To measure the number of *Salmonella* revertants, 1.0 ml of the sonicated *E. coli* preparation, 2.5 µg DMN and 25 µg AAF were incubated for 15 min at 37 °C. The reaction mixture is then mixed in 3.0 ml 0.75% minimal histidine soft-top agar and 10⁸ cells of *S. typhimurium* TA98 of Ames¹ are added. The mixture is poured over minimal agar plates and incubated at 37 °C for 48 h. The mutation ratio is the number of colonies on the experimental plate to the number of colonies on a control plate which is inoculated with the same number of tester bacteria.

*Cell-free extract heated for 15 min in boiling water bath before incubation with DMN and AAF.

†One ml of cell-free extract pre-incubated with 0.1 mg Pronase 20 min before addition of DMN and AAF.

‡DMN (2.5 µg), AAF (25 µg) and 1 ml cell-free extract of *E. coli* were incubated for 15 min. CH₂Cl₂ (5 ml) was added, the mixture shaken, the phases separated after standing 10 min and methylene chloride phase-evaporated to dryness under a stream of N₂; the residue was dissolved in 1.0 ml DMSO and added to 3.0 ml soft-top agar with 10⁸ cells of TA98 and poured over minimal agar plate in a standard Ames test.

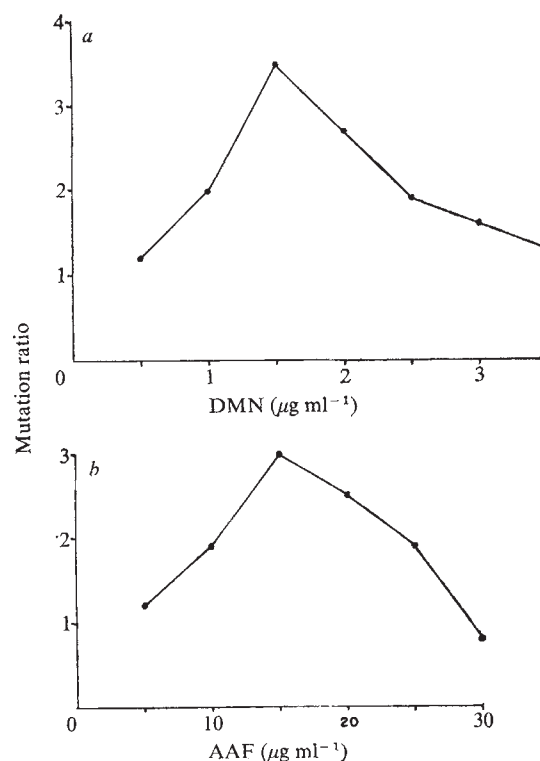
concentration and then decrease with higher concentration of DMN (Fig. 3a). A similar curve was obtained by varying the AAF concentration at constant DMN concentration (Fig. 3b).

Nitrosamides are known not to require activation⁷ by liver microsomal enzymes and are capable of reacting directly with DNA. It is possible that these observations reported here (Figs 1 and 2, Table 1) are the result of a nitroso group exchange reaction in which the nitroso group is transferred from the nitrosamines to the amide moiety of AAF. To gain support for this hypothesis an experiment (Table 2) was carried out in which AAF was replaced with a

Table 2 Mutagenic activity of a cell-free preparation of *E. coli*, DMN, various amides and other substances, using *S. typhimurium* TA98

Compounds of mixture	Mutation ratio TA98
1 AAF+DMN+JC5519 prep	2.8
2 Urea+DMN+JC5519 prep	2.6
3 Urea+JC5519 prep	1.0
4 DL-alanyl-DL-alanine+DMN+JC5519 prep	1.9
5 Glycyl-L-tyrosine+DMN+JC5519 prep	2.3
6 Choractyl-L-tyrosine+DMN+JC5519 prep	2.2
7 DL-alanyl glycine+DMN+JC5519 prep	1.9
8 L-phenylalanyl-L-phenylalanine+DMN+JC5519 prep	2.5
9 N-acetyl-L-tryptophan+DMN+JC5519 prep	2.4
10 Tryptophan+DMN+JC5519 prep	1.0
11 Valine+DMN+JC5519 prep	1.1
12 Lysine+DMN+JC5519 prep	1.0
13 2-Aminofluorene+DMN+JC5519 prep	1.1

Experimental conditions as described in Table 1, except for the substitution of various amides instead of AAF.

**Fig. 3** Mutation ratio as a function of concentration. *a*, AAF held constant at 25 µg ml⁻¹ and DMN varied from 0.5 to 3.5 µg ml⁻¹. *b*, DMN held constant at 2.5 µg ml⁻¹ and AAF varied from 5 to 30 µg ml⁻¹. Procedure as given in Table 1.

series of other amides. Mutagen activity was observed with urea, a series of dipeptides and DMN in conjunction with the sonicated bacterial extract of JC5519, but no revertants were formed when amino acids or aminofluorene were substituted for AAF.

We believe that these observations may have importance in understanding the role of nitrosamines and the cause of cancer. *E. coli* is a common enteric bacteria, widely distributed in the environment and nitrosamines can be formed in a variety of circumstances. In the presence of amides of many types, highly reactive nitrosamides can be generated which may enter adjacent cells and cause mutation and possibly transformation to the neoplastic state. In this regard we have been able to detect in faecal flora the nitroso exchange reaction and we are exploring this reaction as a possible cause of colorectal cancer.

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Mast cell histamine and cell dehydration thirst

RAT mast cells, which synthesise and secrete the dipsogenic amines, histamine and 5-hydroxytryptamine (5-HT), are particularly abundant around small blood vessels and are known to be very sensitive to variations in tonicity¹. Hypotonic solutions cause their massive degranulation²⁻⁴, release amines from their granules^{5,6} and increase urinary histamine excretion⁷. Hypertonic solutions cause mast cell degranulation⁸ and shrinkage⁹. We report here evidence which suggests that the mast cell is an osmolar receptor and that histamine participates in the afferent link of the drinking behaviour elicited by hypertonic NaCl.

Male rats (300–320 g) were housed individually, and maintained on a 12-h dark-light cycle with continuous access to food pellets and tap water, except during the drinking tests when distilled water was used and food was removed. The drinking stimuli were the intraperitoneal (i.p.) injection of 3% NaCl (2.5 ml per 100 g)^{10,11} and the intravenous (i.v.) injection of 2 M NaCl (0.5 ml per 100 g)¹².

It is well known that the intraperitoneal injection of distilled water results in the depletion of mesenteric mast cells, whereupon they are replaced only slowly, regeneration becoming complete only about 6 weeks later^{4,13}. As can be seen in Fig. 1, the administration of 3% NaCl i.p. to rats injected 24 h earlier with distilled water (7.0 ml per 100 g i.p.) to deplete peritoneal mast cells, resulted in a marked reduction in their water intake during the first 2 h, although this difference was considerably reduced after 24 h (Table 1).

These data suggest that the peritoneal mast cell could be involved in the drinking response elicited by the intraperitoneal administration of 3% NaCl, but do not exclude the possibility that this is due to their anatomic disruption by the locally injected hypertonic solution.

The postulation of the peritoneal mast cells as an osmo-receptor requires the demonstration that peritoneal mast cell-depleted animals drink less in conditions of acute dehydration when the hypertonic NaCl load is administered by any other route.

Rats were anaesthetised with ether and the external jugular vein catheterised as described by Fitzsimons¹² for the intravenous injection of hypertonic solutions in unrestrained animals. When hypertonic NaCl was administered by this route, the drinking response was also significantly reduced in rats injected intraperitoneally with distilled water, 24 h earlier (Table 1). In fact, there were no differences between the amounts of water drunk by rats injected intraperitoneally or intravenously with hypertonic NaCl, either in the control or in the peritoneal mast cell-depleted groups.

Assuming that distilled water could alter other cellular structures in the mesentery besides disrupting the mast cells, we used disodium cromoglycate (DSCG), a drug that inhibits the release of histamine from the rat mast cell^{14,15}.

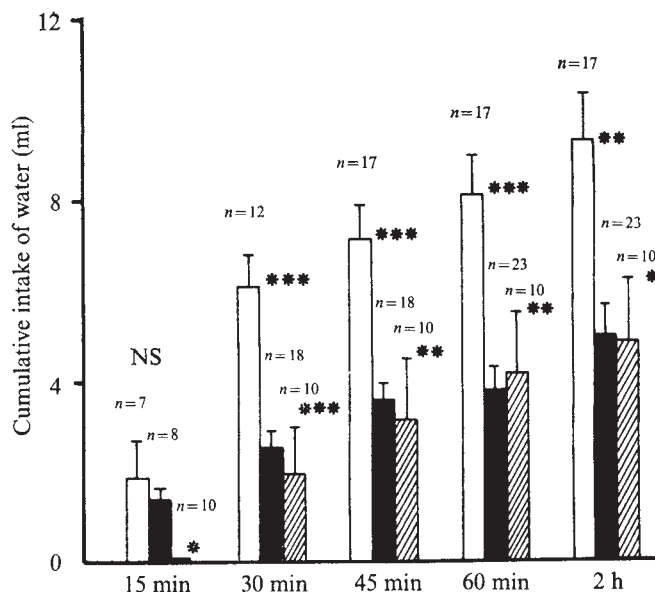


Fig. 1 Cumulative water intake ($\pm$ s.e.) for control rats (light bars), rats pretreated with intraperitoneal distilled water to deplete peritoneal mast cells (dark bars), and rats pretreated with DSCG (striped bars), after the injection of 3% NaCl 2.5 ml per 100 g i.p. * $P < 0.01$; ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0005$. The t test has been applied to control compared with distilled water and control compared with DSCG groups.

The administration of DSCG (95 mg kg⁻¹ i.p.) 5 min before injection of 3% NaCl i.p., resulted in a marked inhibition of drinking, of the same magnitude as that observed with the distilled water pretreatment (Fig. 1). As in the previous experiments, the differences were blurred after 24 h (Table 1). Increasing the dose of the drug (up to 190 mg kg⁻¹) did not result in further inhibition of drinking. Neither distilled water nor DSCG depressed or inhibited the drinking response to other dipsogenic stimuli (Table 2). These results indicate that the peritoneal mast cell is in fact involved in the detection of osmolarity changes in the body fluids.

5-HT exerts its dipsogenic effect through the release of kidney renin and the plasmatic generation of angiotensin¹⁶. As thirst elicited by hypertonic solutions is not mediated by the renal renin-angiotensin system^{10,11,17,18}, it seemed unlikely that mast cell 5-HT would be responsible for the drinking behaviour induced by highly concentrated NaCl solutions. As expected, pretreatment with the 5-HT antagonist, methysergide 2 mg kg⁻¹, which blocks 5-HT receptors without evidence of stimulation¹⁹, did not have any effect on the drinking response elicited by hypertonic NaCl (cumulative intake of water after 2 h: controls, 9.3 ± 1.0 , $n=17$; methysergide, 8.9 ± 0.5 , $n=4$).

On the other hand, intraperitoneally injected histamine induces drinking in intact and nephrectomised rats²⁰, which suggested that the hypertonic NaCl thirst could be mediated

Table 1 Effect of peritoneal mast cell depletion and DSCG on rats injected with hypertonic NaCl

	30 min	60 min	2 h	24 h
3% NaCl i.p.	6.43 $\pm$ 0.63 (22)	8.24 $\pm$ 0.66 (27)	8.98 $\pm$ 0.77 (27)	20.55 $\pm$ 2.17 (11)
2 M NaCl i.v.	8.05 $\pm$ 1.63 (10)	9.55 $\pm$ 1.58 (10)	10.30 $\pm$ 1.46 (10)	26.00 $\pm$ 3.65 (10)
DW + 3% NaCl i.p.	2.63 $\pm$ 0.28 (28)	3.65 $\pm$ 0.38 (33)	4.77 $\pm$ 0.52 (33)	18.20 $\pm$ 2.00 (21)
DW + 2 M NaCl (i.v.)	2.64 $\pm$ 0.88 (11)	3.05 $\pm$ 0.92 (11)	3.77 $\pm$ 1.04 (11)	20.91 $\pm$ 1.63 (11)
DSCG 95 mg kg ⁻¹ + 3% NaCl i.p.	1.95 $\pm$ 1.01 (10)	4.20 $\pm$ 1.39 (10)	4.95 $\pm$ 1.39 (10)	17.20 $\pm$ 3.60 (10)
DSCG 190 mg kg ⁻¹ + 3% NaCl i.p.	2.38 $\pm$ 1.00 (8)	4.75 $\pm$ 1.52 (8)	5.13 $\pm$ 1.40 (8)	16.13 $\pm$ 4.30 (8)

The amount of water drunk (mean $\pm$ s.e.) by rats injected intraperitoneally intravenously with hypertonic NaCl, pretreated with distilled water (DW, i.p.) to deplete mast cells, 24 h earlier, and with disodium cromoglycate (DSCG), 5 min earlier. For experimental details see text. Number of rats given in parentheses.

Table 2 Effect of distilled water, DSCG, and H₁ and H₂ antihistamines on the drinking induced by different thirst stimuli

Pretreatment	Thirst stimuli			
	24-h water deprivation (30 min CIW)	Isoprenaline 0.247 mg kg ⁻¹ s.c. (2 h CIW)	3% NaCl i.p. 2.5 ml per 100 g i.p. (2 h CIW)	Histamine 10 mg kg ⁻¹ i.p. (45 min CIW)
Control	8.5±0.8 (10)	9.0±1.0 (6)	8.9±0.7 (27)	4.2±1.2 (8)
DW 7.5 ml per 100 g i.p.	8.4±0.3 (10)	7.5±1.1 (12)	4.7±0.5 (33)	6.8±1.5 (5)
DSCG 95 mg kg ⁻¹	10.6±1.1 (10)	8.9±1.6 (5)	4.9±1.3 (10)	6.1±0.7 (8)
Diphenhydramine 40 mg kg ⁻¹	0 (10)	0 (10)	0 (10)	0 (10)
Mepyramine 32 mg kg ⁻¹	2.7±1.4 (6)	2.3±2.2 (4)	1.8±0.8 (5)	0 (5)
Metiamide 50 mg kg ⁻¹	10.5±1.0 (10)	7.2±2.2 (7)	2.0±0.4 (7)	1.0±0.3 (7)

The cumulative water intake (mean ± s.e.) of rats pretreated with distilled water (DW) 24 h before the start of the water deprivation period, DSCG i.p. 5 min before, and diphenhydramine, mepyramine and metiamide, 10 min before the administration of the other stimuli and the availability of drinking water. Number of animals given in parentheses.

by histamine released from the peritoneal mast cells. The drinking induced by intraperitoneally injected histamine has been reported to be partially inhibited by the pretreatment with diphenhydramine 40 mg kg⁻¹, an antihistamine drug which blocks H₁ receptors²¹. When we pretreated with diphenhydramine 40 mg kg⁻¹ i.p. 10 min earlier, however, we completely abolished the water intake induced by 3% NaCl i.p., both in normal and nephrectomised animals, after 2 h, and markedly diminished the cumulative water intake after 24 h (Table 2). As it is well known that H₁ antihistamines also show anticholinergic activity^{19,22} and that diphenhydramine is the best known member of this group of drugs, we tried to discriminate between a specific antihistamine effect or a reduction of water intake due to the inhibition of cholinergic synthesis essential in the drinking response¹⁷. As can be seen in Table 2, diphenhydramine also abolished thirst induced by isoprenaline 0.247 mg kg⁻¹ subcutaneously, histamine 10 mg kg⁻¹ i.p., and water deprivation (24 h). These results showed that diphenhydramine is a nonspecific inhibitor of drinking in the rat.

Mepyramine 32 mg kg⁻¹, an H₁ antihistamine which is 40,000 times more active against histamine than acetylcholine²², reduced markedly and to the same extent the drinking induced by 3% NaCl, water deprivation (24 h),

histamine and isoprenaline (Table 2). These data, obtained with two H₁ antihistamines with different amounts of anticholinergic activity, suggested that either H₁ receptors were not involved in the histamine-induced thirst or that the administration of H₁ blockers was useless because they always inhibit drinking by a central effect.

We therefore tried the H₂ receptor blocker, metiamide, which shows practically no penetration into the central nervous system²³. As can be seen in Fig. 2, this H₂ antihistamine did not inhibit water intake induced by water deprivation (24 h) and isoprenaline, but blocked almost totally the drinking induced by 3% NaCl i.p. and histamine (Table 2).

Our experiments show that the reduction in the number of mesenteric mast cells and the blockade of mast cell secretion by DSCG reduce significantly the intake of water following the administration of hypertonic NaCl. This effect is most evident 2 h after the intraperitoneal or intravenous injection of the Na load, but is insignificant after 24 h. This was to be expected, as distilled water only destroys the mesenteric mast cells, leaving the rest of the mast cell complement of the body intact, and DSCG is rapidly eliminated¹⁴.

These experiments suggest that the mesenteric rat mast cell has a role in the detection of changes in tonicity of the body fluids. The mast cell is endowed with some of the characteristics that can be expected to be present in an osmotic receptor: perivascular distribution; high sensitivity to changes in tonicity; the ability to synthesise and release amines which induce drinking behaviour and a peculiar mechanism of secretion which involves the endocytosis of extracellular fluid²⁴⁻²⁶; and the eventual exchange of extracellular Na⁺ for histamine within the intracellular storage granules without exocytosis, as suggested by Foreman *et al.*²⁷.

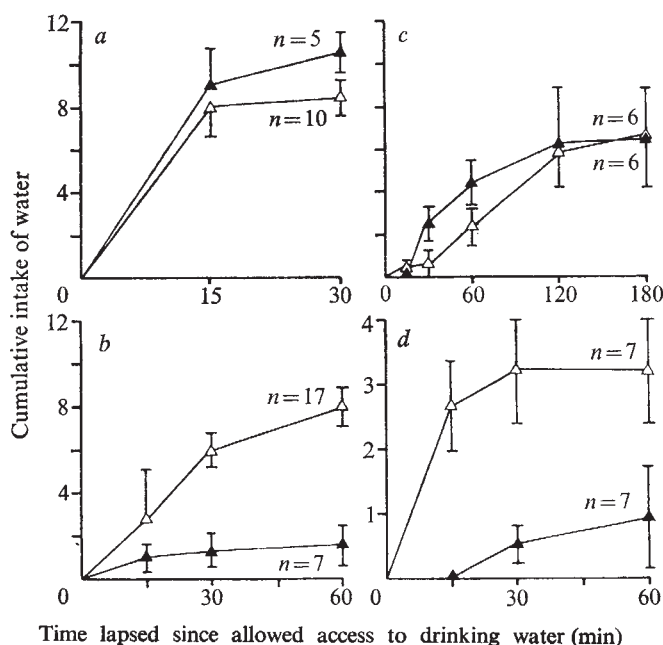
The inability of methysergide to inhibit thirst and its abolishment by metiamide in rats injected with 3% NaCl i.p. indicates that histamine is the mast cell amine involved in the triggering of the drinking response induced by a hypertonic load through the activation of an H₂ receptor.

Histamine induces thirst in the nephrectomised rat²⁰. This shows that the dipsogenic effect of histamine is not wholly mediated by the release of kidney renin and the plasmatic generation of angiotensin, and demonstrates the existence of another mechanism(s) able to convey to the central nervous system specific signals of thirst induction.

Studies using ¹⁴C-histamine have shown that it can cross the brain-blood barrier, even if in limited amounts²⁸. However small, these amounts of histamine might be of physiological importance, since, when applied directly into the hypothalamus, as little as 50 ng histamine can elicit a drinking response²⁹. The fact that metiamide, which does not cross the brain-blood barrier, inhibits both the drinking induced by histamine and hypertonic NaCl i.p., suggests that histamine triggers peripheral stimuli which reach the brain eliciting thirst.

Abdominal vagotomy produces severe deficits in drinking

Fig. 2 Influence of metiamide on the cumulative amount of water drunk by (a) rats deprived of drinking water for 24 h and after the injection of (b) 3% NaCl 2.5 ml per 100 g i.p.; (c) isoprenaline 0.247 mg kg⁻¹ s.c., and (d) histamine 10 mg kg⁻¹ i.p. The vertical bars are twice the s.e. △, Control; ▲, metiamide 50 mg kg⁻¹ i.p. 10 min before the rats were allowed access to drinking water (a) or 10 min before injection of the drugs (b, c and d).



behaviour in response to hypertonic NaCl, that are not reproduced by the administration of peripheral anticholinergic drugs³⁰. These data suggest that the critical lesion is the loss of afferent vagal stimuli. It is tempting to speculate that these vagal afferent stimuli are triggered by histamine, through an H₂ receptor. Experiments to test this hypothesis are in progress.

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Regulation of acid proteases during growth, quiescence and starvation in normal and transformed cells

CELL protein exists in a continual but variable state of turnover and the total protein content is the net result of the relative rates of synthesis and degradation. Cell growth is the net accumulation of protein, and although the relative contribution of each process is still uncertain the cessation of cell growth could be the result of a decrease in synthesis¹ and/or an increase in degradation². We report here that cessation of growth and starvation in normal cells is accompanied by a large increase in acid protease activity;

however, transformed cells which fail to stop growing also fail to elevate the enzymes responsible for hydrolysis of the great majority of peptide bonds.

When the culture medium is partially depleted of either essential nutrients³ or macromolecular serum growth factors⁴, the cell which retains its growth control will cease proliferation, and enter the G₀ (or extended G₁) phase of the cell cycle, where it can survive for prolonged periods in concentrations of these requirements less than those necessary for cell division. Some of the events which have been observed in response to deprivation of either nutrients or serum factors have been collectively called the 'pleiotypic response'⁵, and an analogous set of events in nutrient-deprived bacteria is known as the 'stringent response'⁶. Among the fundamental events in these starvation responses in both prokaryotes and eukaryotes is an increase in the rate of general cell protein degradation^{3,6}; however, the mechanisms of rapid and long term alterations in proteolysis have remained unknown. The function of the elevation in protein degradation during starvation is the maintenance of amino acid pools as a source of metabolic energy and as precursors for essential protein synthesis.

Whereas the rate of protein degradation is increased during cell starvation, there is now ample evidence that it is decreased during cell growth. As determined by the extracellular release of incorporated radiolabelled amino acids from cell protein, decreased proteolysis as well as increased synthesis contributes to the net gain of protein in several cell types, as observed *in vitro* and *in vivo*⁷. In contrast to the case of the increase in proteolysis during starvation, the cellular logic of the decrease during proliferation is uncertain, and space does not permit discussion of possibilities.

It is helpful for purposes of the present investigation to organise the above observations of the eukaryotic cell into three roughly distinguishable levels of protein degradation corresponding to three cellular conditions of proliferation, quiescence under adequate nutrition, and quiescence under starvation. The resting cell under adequate nutrition degrades its protein at a rate of approximately 2–3% h⁻¹ (ref. 7). Reports of this value, the quantitative extent of the decrease during proliferation and increase during starvation, vary considerably among different cells and tissues and are occasionally contradictory due in part to experimental difficulties in measuring protein degradation.

Figure 1 shows the simultaneous variation in cathepsin D activity, cell growth, and thymidine incorporation in 3T3 cells grown with and without changing the medium. When grown without medium change, cells proliferated logarithmically until d4; growth then slowed; and at d8 there was no increase in cell number. Changing the medium on d4 caused the characteristic stimulation of DNA synthesis and cell growth shown in Fig. 1a, and final cell saturation density was six to seven times that of the unchanged cultures. Cathepsin D activity per cell in those cultures in which the fluid was not changed, increased 10–15-fold from its initial value in logarithmically growing cells and increased five to sixfold in those cultures in which the fluid was changed. Stimulation of growth caused the transient decline in protease activity shown in Fig. 1b, the extent of which depends on the persistence of the activity (enzyme half life) as well as the regulation of its synthetic rate. Thus, in the three different cellular condition of logarithmic growth, quiescence under adequate nutrition, and quiescence under a deficit of nutritional or hormonal factors, there are three different levels of the acid proteases (Fig. 1b). The identity of the factors depleted from the medium which cause the elevation in acid protease activity, is being investigated.

Those starving transformed cells which have completely lost their growth control, such as the Simian virus (SV)-transformed SV3T3 cell used in the present investigation, do

not undergo the same orderly alteration in diverse aspects of metabolism, which permits the quiescence and non-proliferative survival of normal cells or those tumourigenic cells which retain the capability to enter the G_0 phase. Instead, they proliferate suicidally; and, during the death of the culture, cells are distributed in all phases of the cell cycle⁸. (*In vivo* tumourigenicity probably involves factors in addition to alteration of proliferative controls.) The SV3T3 cell protease activity was very similar to that of the 3T3 during logarithmic growth; however, in complete distinction from the untransformed cell, the SV3T3 cell activity remained constant even into the death phase of the culture (Fig. 2). The SV3T3 cells did not secrete an appreciable fraction of the total content of the activity into serum-free medium during a period of 10 h; therefore, extracellular secretion is not a major route of turnover of this enzyme in this cell type. Thus, this highly transformed cell, which has lost the ability to enter G_0 in response to starvation, has also completely lost the ability to elevate the enzymes responsible for the bulk of intracellular protein degradation. The observed deficiency in protease regulation could be secondary to loss of some central control mechanism which also governs other aspects of metabolism ('unitary lesion') or alternatively could be an independent event.

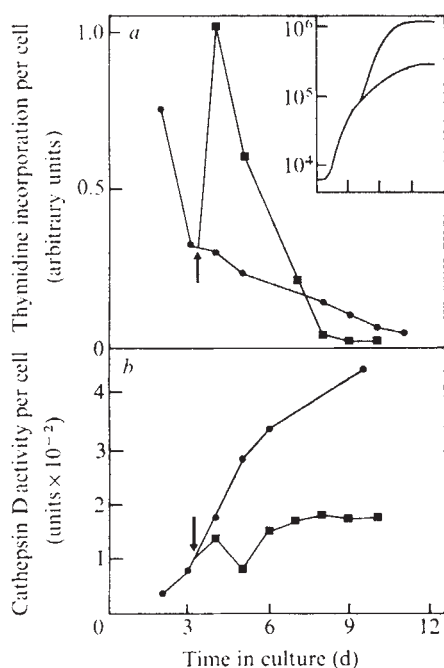


Fig. 1 Simultaneous variation in incorporation of thymidine per cell (a) and cathepsin D activity per cell (b) in BALB/c 3T3 cells compared with time in culture. 5×10^3 cells per 5-cm plate were seeded in Dulbecco-Vogt's modified Eagle's medium containing 10% foetal calf serum, and grown in a humidified incubator. At the time indicated by the arrow, the fluid of one group of plates was changed to fresh medium (●), whereas the other group was cultured in the original medium with no additions (■). At daily intervals, four plates of cells were counted with a Coulter counter (a, insert) and four plates were pulsed with ^3H -thymidine ($1 \mu\text{Ci ml}^{-1}$, 3 μM , New England Nuclear, 6.7 Ci mM^{-1}) for 2 h. Incorporated radioactivity per cell (a) was determined in washed 6% TCA precipitates (a). Cathepsin D activity (b) was determined by triplicate assay of the 0.1% Triton X-100 whole-cell homogenate pooled from four plates as described in Fig. 2. The decline in activity towards the initial value after fluid change was reproducible. Cell viability was 99% at day 10 as determined by Trypan dye exclusion. Increases in activity were somewhat greater when expressed as per unit of cell protein¹⁴.

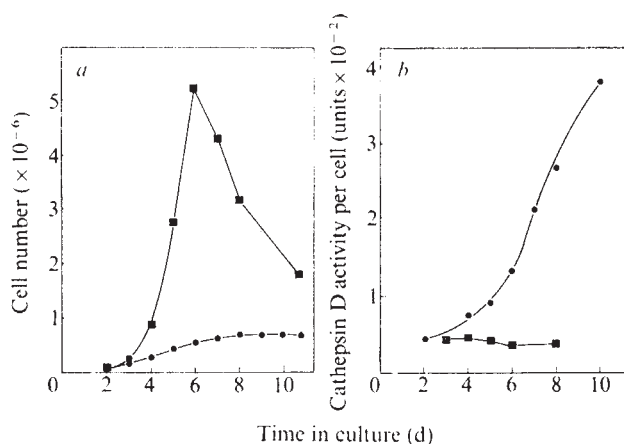


Fig. 2 Cell growth (a) and cathepsin D proteolytic enzyme activity per cell (b) in 3T3 and SV3T3 mouse fibroblasts, compared with time after plating. Both 3T3 and SV3T3 cells were seeded at 10^4 cells per 5-cm plate in the same batch of Dulbecco-Vogt's modified Eagle's medium containing 10% foetal calf serum, and grown without changing the medium. In these conditions the 3T3 cell populations (●) ceased proliferation and entered the G_0 phase of the cell cycle at 6–8 d, whereas the SV3T3 cells (■) continued to divide into the death phase (a). At daily intervals, three plates of cells were rinsed three times with phosphate-buffered saline and collected by scraping in 1 ml 0.1% Triton X-100. Three parallel cultures were counted with a Coulter counter. The cells were homogenised by passage through a fine gauge needle 10 times, and cathepsin D activity was determined in the whole-cell homogenate at pH 2.8 in 0.2 M citrate-phosphate buffer by TCA solubilisation of ^3H -haemoglobin¹⁵. A unit of enzyme activity is equivalent to 1 μg haemoglobin hydrolysed to 6% TCA soluble form per 10^6 cells per hour. At 10 d the viability of the 3T3 cells was greater than 99%. The slight decrease in SV3T3 activity per cell is due to the presence of 5–7% dead cells adhering to the monolayer, and when corrected accordingly, yields a constant value. Cathepsin D activity increased 12–15-fold in primary cultures of BALB/c embryonic fibroblasts and in Swiss 3T3 cells similar to that shown above for BALB/c 3T3 cells (b, ●), but remained constant in the BALB/c SV3T3 cell (b, ■) and Swiss SV3T3 cell. Culture in 0.2% serum also caused no elevation of cathepsin D activity in the SV3T3 cell (data not shown).

The profile of pH against protease activity which contains several proteases in addition to cathepsin D, seemed to be identical in logarithmically growing 3T3 and SV3T3 cells (data not shown). Furthermore, the entire pH against activity profile in 3T3 cells increased in a precisely constant proportion, indicating a regulatory alteration common to at least several acid proteases.

As observed in tissue culture, loss of mitotic control is not an all-or-none phenomenon, and diverse tumourigenic cell lines retain varying extents of the properties usually considered indicative of normal cells. For example, the HTC hepatoma cell under extreme nutritional deprivation exhibits both a G_0 arrest⁹ and an elevation in protein degradation¹⁰, whereas the SV3T3 cell used in these investigations undergoes neither³. Therefore, the regulation of lysosomal proteases was investigated in a benzo(α)pyrene-transformed cell line (BP3T3) which enters $G_{0.1}$ only under extreme serum deprivation^{11,12}. Accordingly it was found that these cells exhibited an intermediate degree of loss of protease control.

The quantitative relationship between alterations in cell content of acid proteases and corresponding alterations in the actual rate of protein degradation is currently unknown. An important precaution in the interpretation of the present data is the fact that control over intracellular protein degradation probably involves several factors in addition to the cell content of lysosomal proteases^{2,13}. Nevertheless, in conjunction with supportive studies of protein degradation, the pronounced elevation of lysosomal protease activities in untransformed cells on quiescence and starvation, and

regulatory deficiency of proteases in transformed cells, imply an involvement of these enzymes in the events of normal and altered proliferative control and altered proliferative control.

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Effect of hormones on herpes simplex virus type-2-induced transformation

GLUCOCORTICOID hormones enhance *in vitro* infections with murine leukaemia virus¹ and mouse mammary tumour virus². The yield of certain strains of herpes simplex virus type 2 (HSV-2) in certain cell types also increases in the presence of glucocorticoid hormones³. But in one limited study no significant differences were observed in the frequency of precancerous and cancerous lesions of the cervix in mice treated with oestrogens and HSV-2 or oestrogen alone⁴. Because there is epidemiological evidence that HSV-2 may be oncogenic in humans⁵, and experimental evidence that inactivated HSV-2 can produce malignant transformation of hamster embryo fibroblast cells *in vitro*^{6,7}, we have studied the effect of different hormones on HSV-2-induced transformation. We have used a rapid quantitative assay for transformation by HSV-2, using a thymidine kinase (TK) selection procedure described elsewhere (ref. 8 and unpublished work with N. Turner). These transformed cells have been shown to contain HSV-specific antigens as determined by indirect immunofluorescence, but do not yield infectious HSV. Our results indicate that 10^{-6} M cortisol and 10^{-5} M oestradiol inhibit HSV-2-induced transformation, and that dexamethasone has very little effect on the incidence of transformation.

A TK⁻ mouse cell line (designated N c1A c110) was obtained from Dr R. Goldberg of the National Institutes of Health and grown in 100-mm Petri dishes in Dulbecco modified Eagle medium supplemented with 10% foetal calf serum (FCS). HSV-2 strain 333 grown in Vero cells was used.

In transformation experiments, virus was ultraviolet-irradiated at $46 \text{ erg s}^{-1} \text{ mm}^{-2}$ and was mixed with trypsinised N c1A c110 cells (6×10^5 cells) at an unirradiated input multiplicity of 2.0 in 2 ml of Dulbecco medium. After adsorption for 1.5 h at room temperature, 15 ml of Dulbecco modified Eagle medium containing 0.16 mM thymidine, 0.05 mM adenosine, 0.1 mM glycine and 0.05 mM guanosine (TAGG medium) was added and the plates were incubated at 37 °C. After 72 h, the medium was removed and 15 ml of MTAGG medium (TAGG medium supplemented with 0.0006 mM methotrexate)⁹ was added. The cells were refed with MTAGG medium 5 d after the original feeding and weekly thereafter. Three weeks after infection, cultures

Table 1 Effect of hormones on HSV-2-induced transformation

Hormone treatment	Average no. of clones per plate	% of control
Untreated control	52	100
Cortisol, 10^{-6} M	10	20
Cortisol, 10^{-6} M	11	20
Cortisol, 10^{-7} M	15	22
Oestradiol- 17β , 10^{-8} M	8	15
Oestradiol- 17β , 10^{-6} M	40	80
Oestradiol- 17β , 10^{-7} M	45	90
Dexamethasone, 10^{-5} M	42	80
Dexamethasone, 10^{-6} M	52	100
Dexamethasone, 10^{-7} M	45	90

HSV-2-induced transformation was measured as described in the text. Hormones were added 4 h after the addition of irradiated virus and remained present for 21 d. Number of clones per plate is average of 4 replicate plates. Mock-infected N c1A c110 cells did not develop clones.

were fixed with 5% formalin and stained with a 1% aqueous solution of crystal violet. Hormones (Sigma) were dissolved in absolute alcohol and added to the medium in appropriate final concentration.

The HSV-2-induced transformation was carried out in the presence of cortisol, oestradiol- 17β or dexamethasone. Table 1 shows a fivefold decrease in the number of transformed clones that developed in the presence of cortisol (10^{-5} to 10^{-7} M). Oestradiol considerably inhibited the number of transformed clones at a concentration of 10^{-8} M, but had no effect at concentrations of 10^{-6} M or lower. In contrast, dexamethasone had very little effect on the number of transformed clones at all concentrations (10^{-5} to 10^{-7} M) examined. Pretreatment of cells with hormones before infection and continuation of hormone treatment after infection (data not shown) did not alter the results described in Table 1.

To determine whether the observed inhibitory effect of cortisol and oestradiol is due to the toxic effect on host cells, N c1A c110 cells were treated with cortisol and oestradiol. After incubation at 37 °C for 48 h, cell numbers and DNA and protein synthesis were measured and compared with untreated controls. Because these cells are deficient in TK, DNA synthesis was measured by determining the incorporation of ^3H -hypoxanthine into alkaline-trichloroacetic acid (TCA)-insoluble material. Table 2 shows that cell numbers, DNA and protein synthesis were not affected by treatment with cortisol or oestradiol at a concentration which inhibited HSV-2-induced transformation. This is further supported by the observation that pretreatment of cells with hormones before the addition of irradiated virus had no inhibitory effect on HSV-2-induced transformation (Table 3).

To determine whether the presence of hormones is

Table 2 Effect of hormones on cell growth and DNA and protein synthesis

	Cell no.* per plate ($\times 10^6$)	DNA synthesis† ^3H -hypoxanthine incorporated (c.p.m.)	Protein synthesis† ^{35}S -methionine incorporated (c.p.m.)
Untreated control	4.80	23,840	59,451
Cortisol, 10^{-6} M	4.89	21,486	61,118
Oestradiol, 10^{-5} M	4.35	22,576	62,198

*Hormones were added 6 h after seeding the N c1A c110 cells into 60-mm Petri dishes. Two days later, both treated and untreated cultures were trypsinised and counted. Number represents average of two replicate plates.

†Hormones were added as described above. One day later, ^3H -hypoxanthine and ^{35}S -methionine were added to both untreated and hormone-treated cells to a final concentration of $1 \mu\text{Ci ml}^{-1}$ and $2 \mu\text{Ci ml}^{-1}$, respectively. After incubation for 48 h at 37 °C, ^{35}S -methionine-labelled cells were lysed with 2% sodium dodecyl sulphate and TCA-precipitable radioactivity was determined. Hypoxanthine-labelled cells were lysed by 0.4% Triton X-100 and digested with 0.3 N KOH at 37 °C overnight, after which TCA-precipitable radioactivity was determined.

necessary throughout the 21 d of incubation, cortisol (10^{-8} M) was kept in the medium for various times and its effect on the number of transformed clones was examined. Data indicate that the presence of cortisol was necessary only for the first 2 d after infection to exhibit inhibitory effect, although inhibitory effect is maximum between 2 and 4 d after addition of virus.

We have examined the possibility that the observed inhibition of transformation is somehow related to a more generalised inhibition of herpes virus development in productive infection. Table 4 indicates that cortisol (10^{-6} M) and oestradiol (10^{-5} M) had no effect on the formulation of herpes virus progeny or on viral DNA synthesis.

Our results demonstrate that HSV-2-induced transformation is inhibited in the presence of cortisol (10^{-5} to 10^{-7} M) and oestradiol (10^{-5} M). In contrast, dexamethasone has very little effect on HSV-2-induced transformation. We do not know why dexamethasone and cortisol, both being glucocorticoid hormones, have different effects on transformation. The difference could be due to the differences in cell receptor for the natural (for example, cortisol) and synthetic (for example, dexamethasone) hormone.

Table 3 Effect of cortisol on HSV-2-induced transformation at various times after infection

Cortisol present (d)	No. of transformed clones per plate
None	55
-2 to 0	50
0 to 2	18
0 to 4	11
0 to 10	10
0 to 21	11
10 to 21	56

Transformation was carried out as described in the legend to Table 1. The interval in which cortisol was present is indicated, zero being the day of virus addition. Twenty-one days after infection, transformed clones were stained and counted. Number of clones per plate is the average of four replicate plates.

Hormones were added 3–4 h after the virus in the transformation assay. We therefore concluded that the inhibition of transformation by hormones is probably not due to the failure of adsorption, penetration or uncoating of infecting virus particles. This was further supported by the fact that the inhibition of transformation was still observed when hormones were added 6–7 h after addition of virus (data not shown). We do not know whether parental viral DNA undergoes replication in the transformation process. Even if the viral DNA is replicated during transformation, this replication probably would not be inhibited by cortisol or oestradiol, because these hormones have no inhibitory effect on viral DNA synthesis in productive infection (Table 4). Because the addition of cortisol at late infection (10 d after infection) did not inhibit transformation (Table 3), it may be concluded that hormones probably do not act at the level of transcription or translation of the virus gene. One possible explanation is that hormones inhibit the integration of HSV-2 DNA into the cellular genome. In the absence of permanent association of viral DNA with the cellular genome, the genetic information for TK is not transferred to the daughter cells, and therefore the formation of transformed clones is inhibited. The mechanism by which hormones might inhibit integration of viral genes into the cellular genome is unknown. This may inhibit the steps involved in integration, for example, specific cleavage of the cellular DNA, circularisation of virus DNA, or joining of virus DNA with cellular DNA. The role of hormones in virus integration has not been reported, but this study may suggest a potential use of hormones to study this

Table 4 Effect of hormones on the synthesis of virus progeny and virus DNA in HSV-2-infected N c1A c110 cells

	Virus yield* (PFU per plate)	DNA synthesis† ³ H-thymidine incorporated (c.p.m.)
Untreated control	4.5×10^6	4.2×10^5
Cortisol 10^{-6} M	4.2×10^6	9.0×10^5
Oestradiol 10^{-5} M	4.3×10^6	3.8×10^5

*Confluent monolayers of N c1A c110 cells were infected with HSV-2 strain 333 at an input multiplicity of 1. After adsorption of virus for 1 h at 37 °C, Dulbecco's modified Eagle medium containing 5% FCS was added. At 24 h after infection, cells were scraped into the medium and the mixture was frozen and thawed three times. After centrifugation at 1,000 r.p.m. for 5 min, the supernatant was assayed for viral progeny in rabbit kidney cells by plaque assay. Numbers represent average of two plates.

†Confluent cells were infected with HSV-2 at an input multiplicity of 5 as described above. At 7 h after infection, ³H-thymidine was added to a concentration of 5 μ Ci ml⁻¹. After 24 h of infection, labelling medium was removed, cells were lysed by 2% sodium dodecyl sulphate and TCA-precipitable radioactivity was determined. Because the CsCl density gradient analysis of labelled DNA synthesised after 7 h of infection is mostly virus specific (our unpublished results), incorporated radioactivity represents virus-specific DNA.

phenomenon. We do not have any direct information about the presence of hormone receptors in N c1A c110 cells, but in conjunction with the accepted mode of action of steroid hormones, we can assume that in this case hormones are also acting through specific cell receptors. There are several possible reasons why we did not see any significant effect of hormones in the productive system. The frequency of integration during the productive infection is presumably very low and any effects would be minimal. Also, productive infection with HSV may change the receptor level in the cells, thereby rendering infected cells insensitive to hormones.

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Cytoskeletal regulation of concanavalin A capping in pulmonary alveolar macrophages

CONCAVALIN A (con A) capping occurs in polymorphonuclear leukocytes (PMN) and is thought to be dependent on disruption of microtubules with subsequent lateral movement of lectin receptors to a polar region in the plasma membrane¹. Capping in PMN is induced by colchicine but is blocked by cytochalasin B, an inhibitor of microfilament assembly². Based on these observations, a cytoskeletal system that effects transmembrane control of receptor mobility has been proposed. The microtubules system interacts directly or indirectly with membrane components to limit the

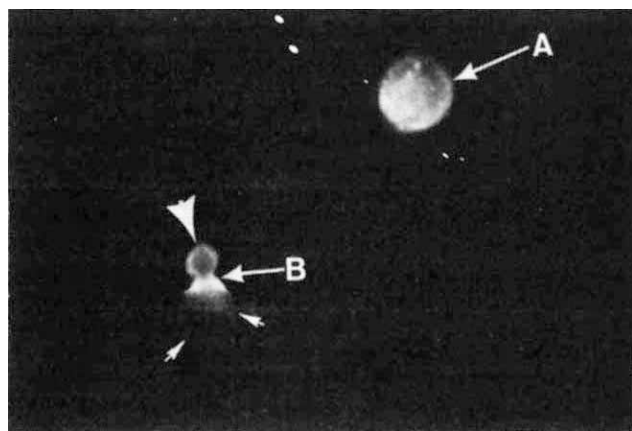


Fig. 1 Photomicrograph of alveolar macrophages showing diffuse fluorescence (A) and polarised fluorescence pattern (cap) (B). Small arrowheads show cell outline, large arrowhead points to fluorescent uropod cap. ($\times 400$.)

mobility of surface receptors while the contractile system of microfilaments may enhance the movement of these receptors into caps³. Here we report that capping also occurs in guinea pig alveolar macrophages (AM), and that the factors which regulate AM surface receptor movement differ dramatically from those in PMN. AM are capped by cytochalasin B alone, which is contrary to the experience with the PMN. AM capping is dependent on oxidative phosphorylation and the cap is associated with formation of a striking uropod (Fig. 1).

Guinea pig AM were obtained using the method of Rister and Baehner⁴, in Krebs-Ringer-phosphate (KRP) buffer with 5 mM glucose. Fluorescein-conjugated con A (FITC-con A, Yeda) was suspended in normal saline. AM were incubated for 2 min at 37 °C; after addition of appropriate drugs (Table 1) for 15 min, AM were labelled with FITC-con A for 5 min at 37 °C, then fixed for 10 min at 28 °C with formaldehyde and centrifuged for 10 min. Slides were prepared and capped cells counted.

Table 1 shows the response of AM to various drugs. Colchicine-induced capping was not inhibited by pretreatment of AM with cytochalasin B ($10 \mu\text{g ml}^{-1}$). Cytochalasin B treatment ($1-20 \mu\text{g ml}^{-1}$) induced an equivalent degree of capping as noted with colchicine. Dimethyl sulphoxide (DMSO), the solvent for cytochalasin B had no effect on

capping. Capping did not occur at 4 °C although warming the AM suspension to 37 °C for 15 min produced maximal capping. AM treated for 45 min with potassium cyanide did not cap, whereas incubation with 2-deoxyglucose, an inhibitor of anaerobic glycolysis⁵ had little effect on capping indicating a dependence on energy derived from oxidative phosphorylation.

Transmission electron microscope studies were carried out to determine the intracellular location of microfilaments and microtubules in capped cells (Fig. 2). In cells treated with colchicine (Fig. 2a), few microtubules were visualised and the cap contained an abundance of microfilaments. In

Fig. 2 Transmittance electron micrograph of alveolar macrophages a, capped after treatment with colchicine in which microtubules are not apparent, and b, after cytochalasin B. A, Outlines the region of cell cap; B, 100 Å filaments; C, microfilament rich region in cap. ($\times 7,000$.)

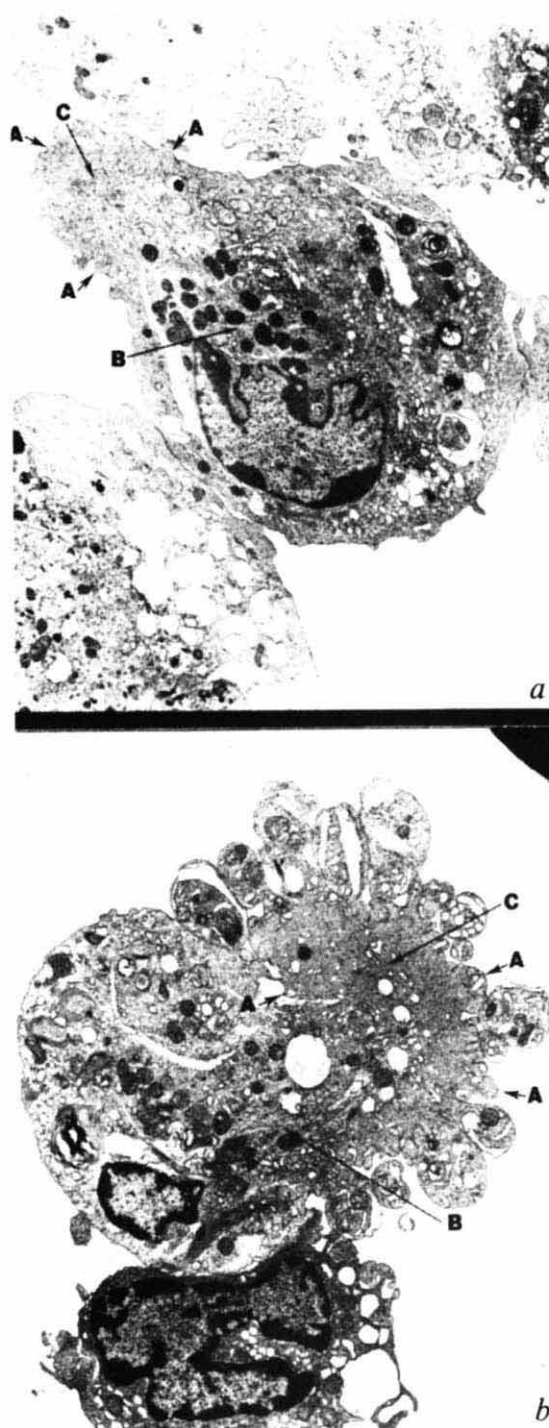


Table 1 Effect of various additions on concanavalin A capping in pulmonary alveolar macrophages

Addition	% Capped
None (control)	31.6 $\pm$ 8.5
Colchicine (10^{-6} M)	63.0 $\pm$ 7.7
Colchicine (10^{-5} M) + cytochalasin B ($10 \mu\text{g ml}^{-1}$)	78.0 $\pm$ 0.9
Cytochalasin B	
1 $\mu\text{g ml}^{-1}$	70.5 $\pm$ 4.3
5 $\mu\text{g ml}^{-1}$	78.6 $\pm$ 7.3
20 $\mu\text{g ml}^{-1}$	73.2 $\pm$ 14.6
DMSO 300 $\mu\text{g ml}^{-1}$	18.2 $\pm$ 6.3
Colchicine (10^{-5} M) at 4 °C	0 0
then warmed to 37 °C	66, 65
Cytochalasin B ($10 \mu\text{g ml}^{-1}$) at 4 °C	0 0
then warmed to 37 °C	70, 73
Potassium cyanide (1 mM)	0 0
2-Deoxyglucose (1 mM)	31, 34

Alveolar macrophages (2×10^6 cells) were incubated at 37 °C in a shaking water bath in KRP buffer, 3 ml total volume, containing 5 mM glucose with drugs indicated. After 15 min cells were stained with FITC-con A ($50 \mu\text{g}$) at 37 °C for 5 min, then fixed with 2% formaldehyde for 10 min at 28 °C. Cells were then washed once and centrifuged and slides made from the cell button. Results were obtained by examining 200 cells per slide at random with a Zeiss fluorescence microscope (FITC-exciter filter, 530 barrier filter) and classifying each cell as capped or not capped. Values represent means $\pm$ s.d.

cells treated with cytochalasin B (Fig. 2b), the caps were surrounded by typical cytochalasin B polarised blebs⁶, but the caps in these cells were also rich in microfilaments.

This study shows that con A capping in the AM can be induced by inhibitors of both microtubule and microfilament assembly. Thus in AM, some microfilaments and microtubules do not seem to have opposing roles in controlling membrane fluidity, as some recent theories propose for other cell types³. Instead, it seems that in the AM, redistribution of lectin receptors, as occurs in capping, could be dependent on a set of microfilaments that are not sensitive to cytochalasin B. Other studies have proposed such a set of microfilaments in the AM (ref. 7). The receptor mobility in the AM may be limited by both the microtubules, as in other cell types, and a subset of microfilaments sensitive to cytochalasin B.

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α -Fetoprotein induces suppressor T cells *in vitro*

THE ability of α -fetoprotein (AFP) of mouse or human origin to suppress certain T cell-dependent immune reactions *in vitro* is well documented¹⁻⁴. These findings on a tumour-associated embryonic substance⁵ have potentially important implications as to our understanding of the maternal-foetal immunological relationship⁶, the development of immune capabilities in the foetus and newborn⁷, and of certain diseases⁸ where immunological hyporeactivity and elevations in AFP often occur concomitantly. The mechanism(s) through which AFP is exerting its immunoregulatory influence is largely unknown; however, previous studies¹ have shown that AFP must be added at the initiation of antigen-stimulated spleen cell cultures for maximal inhibition of primary antibody synthesis to occur.

Continued presence of AFP in the cultures for 8-12 h was sufficient to maintain suppression in an AFP-free environment for at least 5 d *in vitro*¹ and 10 d *in vivo* using adoptive transfer experiments (unpublished). These observations suggested that AFP may inhibit immune responses indirectly by activating regulatory suppressor cells. Here we report that AFP does indeed induce the formation of highly efficient suppressor T cells with capacity to inhibit helper T cells, but with no effect on B cells responding to thymus independent antigens.

Mouse amniotic fluid (MAF) was collected from pregnant outbred Swiss mice (Anticimex, Stockholm) between day 14 and day 16 of gestation. AFP was isolated from MAF by a two-step procedure of affinity chromatography and preparative polyacrylamide gel electrophoresis as described before¹. Purity was checked by gel electrophoresis and immunological tests. In initial experiments normal adult C57BL/6 or BALB/c spleen cells were cultured in Marbrook chambers⁹ with purified AFP, MAF or normal mouse serum (NMS) at equivalent final concentrations in the cultures of 200 μ g ml⁻¹ for 4 d. There were no significant differences in the yields of viable lymphocytes from cultures containing AFP, MAF, and NMS. The precultured cells were then washed, and 10⁶ cells were added to 20 $\times$ 10⁶ fresh normal spleen cells in new Marbrook cultures along with an optimal immunising dose of sheep red blood cells (SRBC). After 4 d in culture the cells were harvested and assayed for primary plaque-forming-cells (PFC) to SRBC. The results of three such experiments are summarised in Table 1. In all experiments the addition of spleen cells precultured in MAF or MAF-derived AFP to assay cultures caused significant suppression of the PFC response to SRBC.

The time-course of development of suppressing ability was determined by assaying for suppressor cell activity after various preculture times ranging from 6 to 96 h. Because of the limited amount of purified AFP available, these and subsequent experiments to be described in this report were performed with MAF. It is justified in this system to attribute the resulting effect to AFP since the previous experiments (Table 1) showed that equivalent suppressor cell activity is obtained by preculturing with AFP and MAF. It has also been clearly demonstrated¹ that the factor in MAF responsible for its suppressive effect on T cell-dependent immune responses is AFP. Although some suppression was evident after 2 d of preculture, 3-4 d were required for maximum development of suppressor cell activity (Table 2). This suggests that AFP is actively generating suppressor cells through a process involving cell proliferation and/or differentiation. In line with this are findings in another test system that AFP under certain culture conditions will induce proliferation of normal mouse T lymphocytes (A. B. Peck, R. A. M. and H. W., in preparation).

Table 1 Suppression of the primary PFC response to SRBC by spleen cells precultured in AFP

Preculture* Cells precultured in:	No. of precultured cells added	Assay culture† γ M PFC per culture (% suppression)		
		Experiment 1	2	3
—	—	1,800	1,640	900
NMS	10 ⁶	2,340 (0)	2,000 (0)	1,116 (0)
AFP	10 ⁶	850 (53)	510 (69)	370 (59)
MAF	10 ⁶	1,000 (46)	420 (74)	573 (36)

*20 $\times$ 10⁶ normal mouse spleen cells were precultured with 200 μ g ml⁻¹ NMS (normal mouse serum), AFP (α -fetoprotein) or MAF (mouse amniotic fluid) in Marbrook chambers⁹ using RPMI 1640 medium supplemented with 10% foetal calf serum, 10 mM HEPES and 5 $\times$ 10⁻⁵ M 2-mercaptoethanol. After 4 d of incubation at 37 °C in 5% CO₂ humid atmosphere, the cells were collected and viable cells were counted by a dye exclusion method.

†10⁶ Viable lymphocytes precultured as indicated above were added to assay cultures of 20 $\times$ 10⁶ fresh normal spleen cells stimulated with 3 $\times$ 10⁶ SRBC in Marbrook chambers⁹. One set of control cultures consisted of fresh spleen cells and antigen without added precultured cells. Direct anti-SRBC PFC were assayed after 4 d in culture². The data from three representative experiments are expressed as arithmetic means of three cultures per group.

Table 2 Time course of development of suppressor cells *in vitro*

Cells precultured in:	Preculture* Duration of preculture (h)	Assay culture† γM PFC per culture	% Suppression
NMS	6	935 ± 45	6
MAF		1,019 ± 214	0
NMS		1,171 ± 158	0
MAF	12	1,197 ± 145	0
NMS		1,092 ± 73	0
MAF		1,095 ± 125	0
NMS	24	887 ± 174	11
MAF		638 ± 89	36
NMS		918 ± 56	8
MAF	48	560 ± 65	44
NMS		1,000 ± 91	0
MAF		372 ± 41	63

*Preculture conditions were as described in the legend to Table 1. The duration of the precultures varied from 6–96 h as indicated.

†10⁶ Viable lymphocytes from preculture were added to assay culture stimulated with SRBC as described in Table 1. Control cultures (cells precultured in NMS) in the experimental group receiving 96 h precultured cells were arbitrarily adjusted to 1,000 PFC per culture. All other values were normalised to these control cultures. Each value represents the mean and s.e.m. of triplicate cultures.

Results presented in Table 3 suggest that the suppressor cell activity induced by AFP is mediated by T lymphocytes. Here the degree of suppressor cell activity generated in precultures of unfractionated spleen cells was compared with that generated by splenic T cells isolated by affinity fractionation on glass bead columns¹⁰. There was a significant enrichment of suppressor activity in precultures of purified T cells, whereas suppressor activity was absent in precultures of spleen cells from nude (*nu/nu*) mice with a BALB/c background, but present in precultures of normal BALB/c spleen cells (Table 3).

A dilution analysis of the suppressor T-cell activity was then performed by adding graded numbers of precultured cells to assay cultures of 20 × 10⁶ normal spleen cells which were then challenged with SRBC or with the thymus independent antigen DNP₃₂-Ficoll¹¹ (DNP, 2,4-dinitrophenyl), and assayed for PFC to SRBC or TNP (2,4,6-trinitrophenyl). A linear dilution effect of AFP-induced suppressor cell activity on SRBC PFC responses was obtained within the range of 10⁷–10⁴ added cells as shown in Fig. 1b. As few as 10⁴ suppressor cells (0.05% of total cell population) markedly inhibited T cell-dependent anti-SRBC responses. There was, however, no effect of suppressor cells on the antibody response to DNP₃₂-Ficoll (Fig. 1a). A similar pattern of suppression of T cell-dependent antibody responses

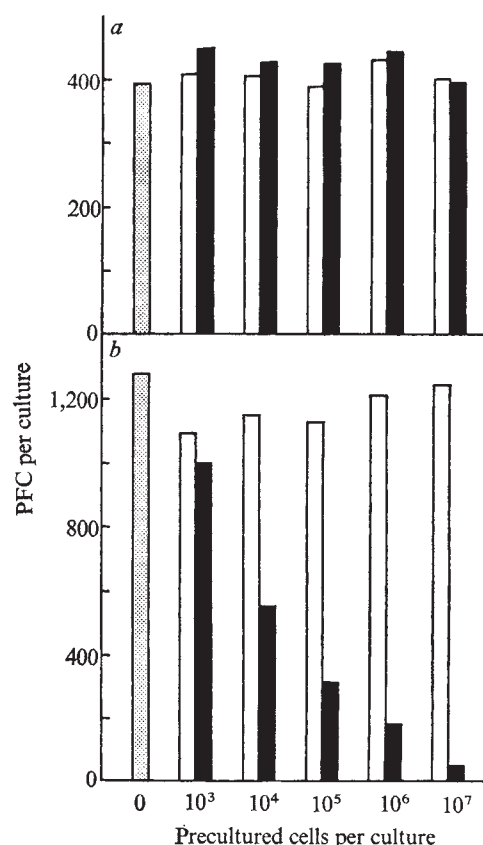


Fig. 1 Effects of various numbers of precultured cells on the primary *in vitro* antibody response to 10 µg ml⁻¹ DNP₃₂-Ficoll (a), and 3 × 10⁶ SRBC (b). The abscissa denotes the number of viable precultured cells added to standard assay cultures. Grey bars refer to antigen-stimulated control cultures without added precultured cells. Open bars refer to cells precultured in NMS and black shaded bars refer to cells precultured in MAF. The thymus-independent PFC response to DNP₃₂-Ficoll¹¹ was assayed against TNP-SRBC².

to DNP-KLH, and a lack of inhibition of T cell-independent antibody responses to DNP-POL has been recorded in several, additional experiments.

Thus our results strongly suggest that one mode of immunoregulatory action by AFP is the generation of suppressor T cells which control the level of responsiveness to T cell-dependent antigens. This is quite in agreement with the findings that in newborn mice with high intrinsic concentrations of AFP there exist efficient numbers of sup-

Table 3 Evidence that T cells mediate suppressor activity *in vitro*

Cell source	Preculture* Cells precultured in:	No. of precultured cells added	Assay culture† IgM PFC per culture	% Suppression
C57BL/6 whole spleen cells	NMS	10 ⁶	1,000†	—
C57BL/6 whole spleen cells	MAF	10 ⁶	990 ± 50	1
C57BL/6 column Purified T cells	NMS	10 ⁶	453 ± 17	55
C57BL/6 column Purified T cells	MAF	10 ⁶	981 ± 32	2
Purified T cells	MAF	10 ⁶	52 ± 8	95
BALB/c spleen	NMS	10 ⁶	1,000†	—
BALB/c spleen	MAF	10 ⁶	1,116 ± 50	0
<i>nu/nu</i> spleen	NMS	10 ⁶	411 ± 78	59
<i>nu/nu</i> spleen	MAF	10 ⁶	1,174 ± 117	0
<i>nu/nu</i> spleen	MAF	10 ⁶	1,117 ± 61	0

*Normal spleen cells, Ig-anti-Ig column purified splenic T cells¹⁰, or spleen cells from thymus-deficient (*nu/nu*) mice were precultured in NMS or MAF for 4 d as described in Table 1.

†The SRBC PFC responses in control cultures of fresh normal spleen cells without added precultured cells were adjusted to 1,000 and experimental values were normalised as described in Table 2.

pressor T lymphocytes^{12,13}. Also, it is known that thymus-derived lymphocytes from newborn humans can function as suppressor cells with regard to the proliferative capacities of the lymphocytes from their mothers (ref. 14 and L. B. Olding, R.A.M. and H. W., in preparation). We therefore suggest that AFP serves as an agent to protect the mammalian foetus from graft-versus-host and host-versus-graft reactions through the selective induction of suppressor T cells. The exact mechanism(s) through which AFP is exerting this activity is now a major target of analysis.

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Unmasking of thromboxane A₂ synthesis by ureteral obstruction in the rabbit kidney

THE rabbit kidney has a high capacity for prostaglandin biosynthesis. The major renal prostaglandin released in response to various stimuli is PGE₂, a vasodepressor prostaglandin¹ thought to have a role in the regulation of intrarenal blood flow². Although renal prostaglandin synthesis may initiate a reduction in vascular resistance of the kidney in response to reduced perfusion pressure, it has not been possible to establish a functional role for renal prostaglandins as mediators of increased vascular resistance evoked by augmented renal perfusion pressure. An increase in renal resistance, if related to changes in the rate of synthesis of endogenous substances, implies the biosynthesis of a vasoconstrictor or suppression of the basal production of a vasodepressor material. Unilateral ureteral obstruction (model of hydronephrosis) in the dog, rat and rabbit is initially associated with a decrease in renal resistance which is followed by a fall in GFR and renal blood flow^{3,4} by 24 h post-occlusion. The initial renal vasodilator phase may be associated with enhanced renal basal PGE₂ synthesis and markedly facilitated PG release in response to hormone stimulation⁵. Here we report the observation that the ureter-obstructed kidney may be producing a natural vasoconstrictor material.

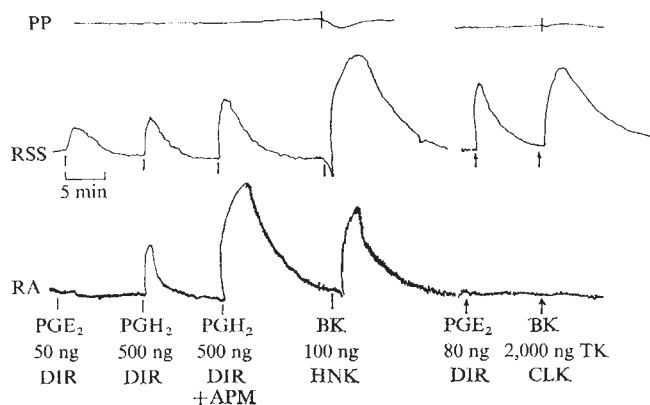
New Zealand male white rabbits were anaesthetised with pentobarbital and the left ureter was ligated⁶. Three days later the rabbits were anaesthetised and both kidneys (ipsilateral ureter obstructed and contralateral untouched kidney) were removed and perfused (15 ml min⁻¹) with Krebs–Henseleit buffer at 37 °C as previously described⁵. The venous effluent was allowed to flow over a series of biological assay organs (rabbit aorta, chick rectum, rat stomach) which were selected to

detect the presence of PGE, PG-endoperoxides and thromboxane-like substances (details Fig. 1 legend).

The isolated perfused rabbit kidney, with the ligated ureter, released into the venous effluent a substance which contracted the rabbit aorta following the administration of a dose of bradykinin (100 ng) (Fig. 1). The contralateral kidney did not release the vasoconstrictor into the effluent even with very high doses of bradykinin (2,000 ng). Alterations in bradykinin metabolism were not enough to explain differences in release from hydronephrotic and contralateral kidney⁵. Indomethacin, a known inhibitor of the cyclo-oxygenase⁸, abolished the generation of the aortic constrictor by the ureter obstructed kidney in response to bradykinin. The PG products of cyclo-oxygenase known to constrict the rabbit aorta are the endoperoxides, PGH₂ and PGG₂ (half life 6–7 min at 37 °C) and thromboxane A₂ (half life 30 s)^{9,10}. The endoperoxides, which are moderately potent aorta vasoconstrictors, are rapidly converted to the classical prostaglandins (PGE₂, PGD₂) by enzymic and non-enzymic breakdown. The enzyme thromboxane synthetase converts the endoperoxide PGH₂ to the potent constrictor thromboxane A₂ and has been demonstrated in platelets⁹, spleen¹⁰ and lung¹¹. Thromboxane synthetase has not been described in the kidney.

To ascertain which of these two prostaglandin constrictors was being produced in the hydronephrotic kidney, the half-time of disappearance of the constrictor substance from the aqueous media was estimated by bioassay. The venous effluent released in response to hormone stimulation was collected, reoxygenated and reperfused over a second aortic strip after a delay of 1.5 min. The original effluent contains both PGE₂ (that is contraction of chick rectum and rat stomach) which is stable in aqueous solution at 37 °C and the rabbit aorta constricting substance (Fig. 2). Following the 1.5 min delay the PGE₂ activity was still present but the constrictor substance had completely disappeared. PGH₂ injected directly over the strips and subjected to the same delay of 1.5 min still produced contraction of the aorta. This instability in aqueous solution suggested that the constrictor was the more labile thromboxane A₂ and not the endoperoxide PGH₂. The renal release of rabbit aorta constrictor activity was dose dependent and was also released from the hydronephrotic kidney following stimula-

Fig. 1 The release of a rabbit aorta contracting substance from a ureter obstructed kidney by bradykinin. The venous effluent from the perfused rabbit kidney is allowed to superfuse a rabbit aortic strip and a rat fundal strip. Sensitivity and selectivity of the assay tissues were increased by an infusion of antagonists⁶ to histamine, acetylcholine, catecholamines and 5-hydroxytryptamine as well as indomethacin (1 µg ml⁻¹) to prevent endogenous synthesis of prostaglandins by the tissues⁷. PP, renal artery perfusion pressure; RSS, rat (fundic) stomach strip; RA, rabbit aortic strip; DIR, injection directly over strips; TK, through the kidney; BK, bradykinin, PGH₂, endoperoxide; HNK, hydronephrotic or ureter obstructed kidney; and CLK, contralateral or untouched kidney (*n* = 10).



tion with angiotensin II 50 ng but not from contralateral kidney with doses up to 1,000 ng (data not shown).

Microsomal fractions from kidney homogenates were prepared for the radiochemical verification of thromboxane synthesis in the hydronephrotic kidney. The kidneys were minced and homogenised (Polytron) in 3 volumes of 100 mM phosphate buffer pH 7.5. The homogenate was centrifuged (8,000g, 15 min) and the supernatant was centrifuged at 100,000g for 60 min. The pellet was resuspended in 100 mM phosphate buffer pH 7.5 in a volume equal to one fourth original wet weight of the tissue. The kidney microsomes (2 mg protein per ml) were incubated with ^{14}C -arachidonic acid in the presence of 1.2 mM L-adrenaline¹³ for 30 min at 37 °C and the volume of the reaction mixture was 1 ml. The reaction was acidified to pH 3.5 with citric acid and extracted twice with 2 volumes of ethyl acetate. The ethyl acetate phase was evaporated to dryness and reconstituted in chloroform-methanol (2:1) and applied to silica gel thin layer plates in a solvent system of benzene-dioxane-acetic acid (60:30:3). The radiochromatogram obtained from the incubation of kidney microsomes and ^{14}C -AA shows radioactive peaks corresponding to PGE_2 , $\text{PGF}_{2\alpha}$, PGD_2 and TXB_2 (thromboxane B_2). Similarly, using another solvent system (chloroform-methanol-acetic acid-water, 90:8:1:0.8) a thromboxane B_2 peak was detected between $\text{PGF}_{2\alpha}$ and PGE_2 . Thus, using two different solvent systems it is possible to demonstrate an arachidonate product which has chromatographic mobility similar to that of thromboxane B_2 . More specific proof of synthesis was obtained by performing the enzymic incubation in the presence of imidazole, a specific antagonist of thromboxane synthetase¹⁴. The thromboxane B_2 peaks were abolished when the renal microsomes were reacted with arachidonate in the presence of 5 mM imidazole (Fig. 3). Similar results, that is, thromboxane B_2 formation which was inhibitable by imidazole, were obtained using ^{14}C - PGH_2 as substrate (instead of ^{14}C -arachidonic acid) with kidney microsomes at 0 °C for 20 min. Only the ureter obstructed kidney seems to possess thromboxane synthetase activity since identical experiments carried out with microsomes prepared from the contralateral unobstructed kidney did not show evidence of a thromboxane B_2 peak on radiochromatograms. These experiments indicate that the microsomal preparation from rabbit kidneys after 3 d of ureteral occlusion can synthesise thromboxane A_2 from arachidonate or the endoperoxide PGH_2 . The ureter-obstructed kidney converted 2.5–3% of the total arachidonate radioactivity to

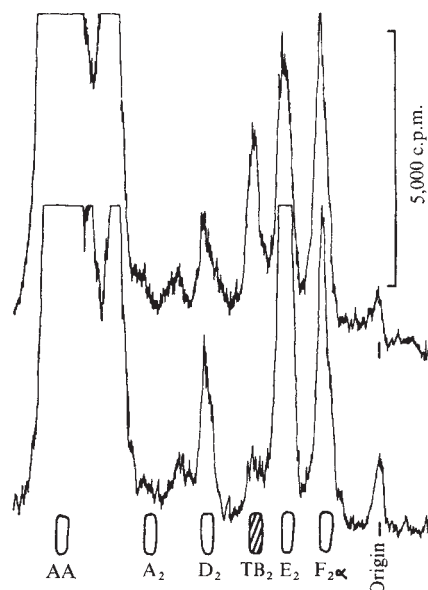


Fig. 3 Thin-layer radiochromatograms of the acid-lipid extract obtained from rabbit kidney microsomes and ^{14}C -arachidonic acid. The upper scan shows the peak of radioactivity between PGE_2 and PGD_2 corresponding to TXB_2 . The lower scan shows loss of this peak when incubations were carried out in the presence of imidazole 5 mM. The mobility of the cold standards in the solvent system of benzene-dioxane-acetic acid (60:30:3) are indicated on the scans. Standards used were $\text{PGF}_{2\alpha}$ ($\text{F}_{2\alpha}$), (E_2) PGE_2 , PGD_2 (D_2), PGA_2 (A_2) and arachidonic acid (AA) (all gifts from Upjohn). TB_2 , thromboxane B_2 ; APM, aspirin treated platelet microsomes ($n = 5$).

^{14}C TXB_2 . The percentage ^{14}C radioactivity converted to PGE_2 , $\text{PGF}_{2\alpha}$ and PGD_2 were 15%, 10% and 5% respectively. Assuming lower rates of conversion for the contralateral kidney, the amount of thromboxane B_2 formed may be below the lower limit of our discrimination.

The unmasking of thromboxane synthetase activity in the experimental model of ureteral obstruction suggests that the biosynthesis of thromboxane A_2 may be important to the alterations in renal vascular resistance and regional blood flow. The potential role of thromboxane A_2 in ureteral obstruction, if it is produced in other species, may be in the alterations in regional blood flow to non-functioning areas of the kidney or decreases in total renal blood flow to a non-functioning kidney in a pathological situation. The capacity to unmask renal thromboxane synthetase activity may give new insight into some old problems.

This work was supported by the NIH.

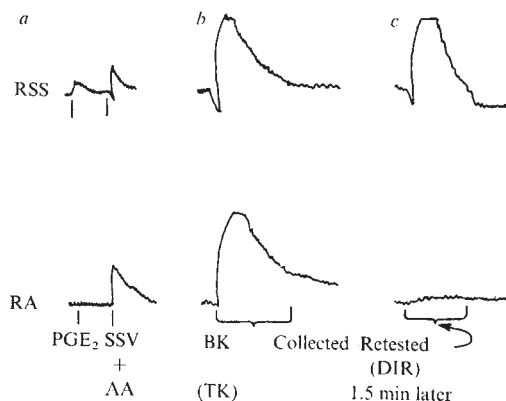
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Fig. 2 Bioassay of rabbit aorta constricting substance (RCS). a, Response of the assay strips, rat stomach strip (RSS) (top) and rabbit aortic strip (RA) (lower trace) to PGE_2 160 ng directly over the strips (DIR) and endoperoxide generated from sheep seminal vesicles (SSV) and arachidonate (AA)¹². b, Contractions of RSS and RA to 200 ng bradykinin (BK) through the kidney (TK). This effluent was collected, re-oxygenated and reperused over a second aortic strip, shown in (c). c, Trace showing complete loss of contractile activity on rabbit aorta after 1.5 min but persistence of PGE_2 activity as indicated by contraction of RSS ($n = 10$).



Is yawning a cholinergic response?

BARBIZET¹ was perhaps right in suggesting that the commonplace, ubiquitous and apparently non-significant nature of the act of yawning, might have contributed to the relative lack of attention given by physiologists to this behavioural phenomenon. The physiological significance of yawning and the nervous mechanisms triggering and coordinating its various components remain unclear^{1,2}. Frequent yawning has been mentioned as a symptom in some diseases of the central nervous system, particularly in cases of frontal tumours and encephalitis^{3,4}. A stretching and yawning syndrome has been observed in cats, dogs and monkeys after intracisternal or intraventricular injections of adrenocorticotrophic and melanotrophic hormones, or synthetic polypeptides with ACTH and/or MSH activities^{5,6}, but the relation of these observations to the basic mechanism of yawning has not been studied. During experiments designed to explore cholinergic potentiation of *D*-amphetamine induced head-shaking in infant rats⁷, we have made some observations that indicate the involvement of a central cholinergic response in yawning.

Infant rats, from 6 h after birth up to 14 d old, were injected intraperitoneally (i.p.) with physostigmine salicylate (0.10 mg of the free base per kg body weight, dissolved in 0.9% NaCl to a total volume of 0.01 ml per g bodyweight). The rats began to yawn about 5 min after the injection. In the following 15 min the number of yawns reached an average of 10–12 during the first 10 d of life. Some animals yawned twice per minute, and others only a couple of times in 15 min. In rats aged under 10 d physostigmine induced yawning was practically universal. Between days 10 and 14 of life the frequency of yawning fell rather steeply, to a level of only 2 or 3 yawns per 15 min, with some animals showing no yawning response. At the 14th day yawning occurrence had decreased to 69% of the population. Each yawn was preceded by salivation, licking of the forepaws and cleaning movements of the snout, or 'gumming' or chewing movements, and sometimes by a sudden stretching of the forelimbs. The yawn itself was a slow wide opening of the mouth, with marked protrusion of the tongue, and lasted about 3–4 s (Fig. 1).

A dose-effect graph of physostigmine induced yawning in

Fig. 1 Physostigmine-induced yawning in a male rat (3 months old) injected with physostigmine at 0.10 mg per kg body weight.

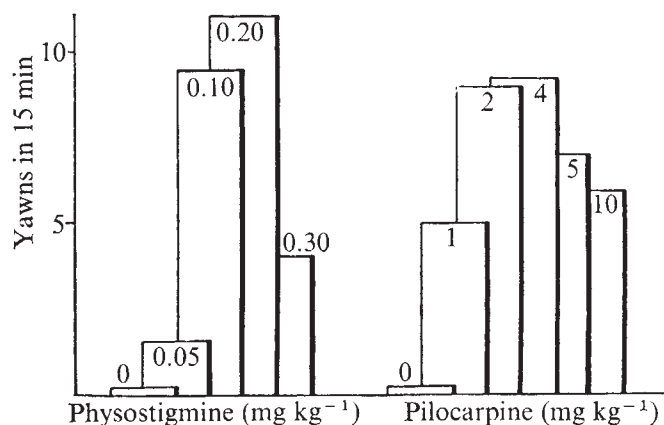
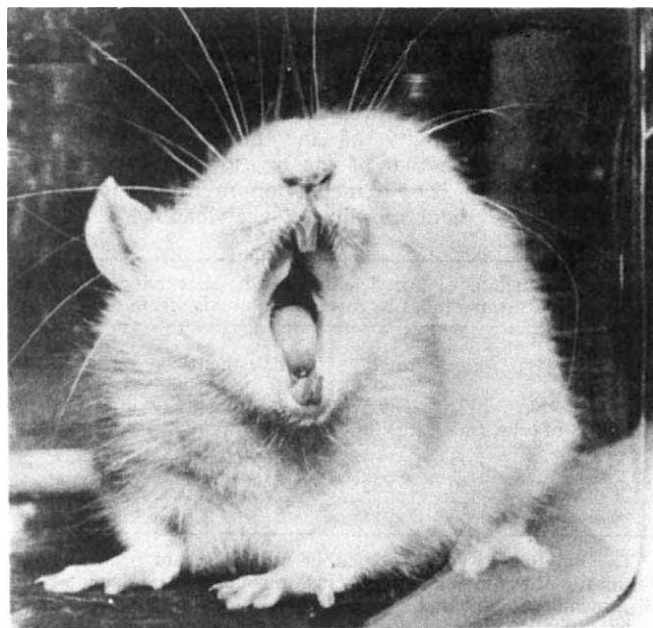


Fig. 2 Physostigmine and pilocarpine induced yawning. Drug doses (free base) are indicated in each column (mg per kg body weight). Each group consisted of 12 nine-day-old rats. All values after drug injection are significantly different from the controls as follows: physostigmine, 0.05 mg, $P < 0.05$; 0.10 and 0.20 mg, $P < 0.001$; 0.30 mg, $P < 0.003$; pilocarpine, all doses tested, $P < 0.001$ (Mann-Whitney U test⁸).

9-d-old rats is shown in Fig. 2. That this particular action of the anticholinesterasic drug is centrally mediated is strongly suggested by control experiments with neostigmine methylsulphate, which passes the blood-brain barrier with difficulty. When injected i.p., in a dose of 0.2 mg per kg, neostigmine induced only 1.6 yawns per 15 min (12 animals). Although this effect is significantly different from that observed in rats injected with saline ($P < 0.01$) it was only of the order of one-sixth of the effect obtained with physostigmine. With higher doses of neostigmine no yawning was observed. Bilocarpine hydrochloride, a cholinomimetic drug that passes readily into the brain, induced yawning up to the same level as physostigmine (Fig. 2).

Cholinomimetically induced yawning seems to be due to the stimulation of central 'muscarinic' receptors, because it is rapidly and completely blocked by scopolamine hydrobromide, at doses of 2.5 to 5 mg per kg (Fig. 3). That 'nicotinic' cholinergic receptors are not participating was demonstrated with nicotine administered by i.p. injection. Doses of nicotine (0.1 mg per kg) which induced clear central effects on head-shaking (unpublished observations) did not induce yawning.

We observed physostigmine induced yawning at a more or less stable level of 2–3 yawns per 15 min in rats from 14 to 90 d of age, but practically only in males. In a pool of 62 animals (31 females and 31 males, 14 to 54 d old) from the same litters, males performed an average of 3.1 yawns in 15 min after physostigmine injection, whereas females performed 0.5 yawns in the same period. The difference is highly significant ($P < 0.002$) by the Mann-Whitney U test⁸. It may be recalled that Hall⁹, in his interesting description of the behaviour of monkeys towards mirror-images, in which 'yawning' or 'gaping' responses were very frequent, also observed that adult females 'rarely, if ever, yawned'.

Our results suggest that in the rat there is a central cholinergic mechanism, with 'muscarinic' receptors, underlying the act of yawning. The mechanism matures at a very early age, suggesting that it is localised at the lower levels of the brain stem. Later development of higher placed cholinergic mechanisms may determine that other effects evoked by cholinomimetic agents injected systemically might be hindering the expression of yawning in older rats. Intense and continuous scratching, or tremor and rigidity, as produced respectively by pilocarpine and physostigmine in rats aged over 10 d, or some other central or peripheral

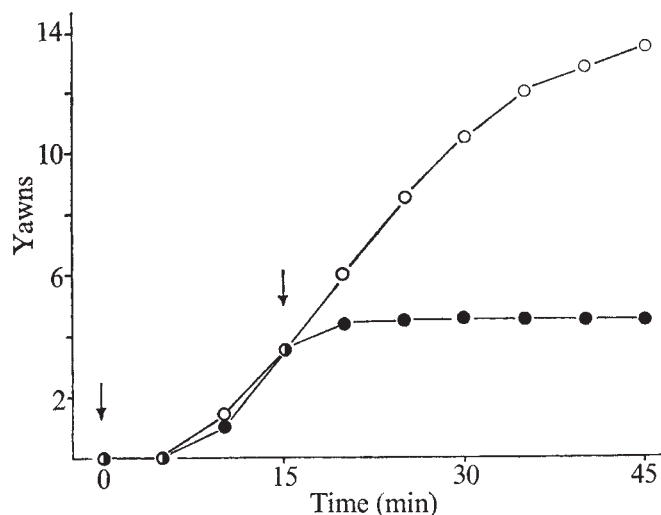


Fig. 3 Blocking effect of scopolamine on physostigmine induced yawning. Arrows indicate the times of injections: at 0 time, physostigmine 0.10 mg per kg; at 15 min, scopolamine 5 mg per kg. The blocking effect of scopolamine is highly significant, $P < 0.001$ already 10 min after injection (Mann-Whitney U test⁸). Similar results were observed in another experimental series with scopolamine, 2.5 mg per kg.

actions affecting the level of vigilance, might be partially counteracting the yawn-inducing effect.

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Myasthenia gravis serum reduces acetylcholine sensitivity in cultured rat myotubes

MYASTHENIA gravis is a neuromuscular disorder characterised by muscle weakness and fatigability. Muscle fibres from myasthenic patients have reduced amplitudes of endplate potentials and miniature endplate potentials^{1,2}. Early studies concluded that the disorder was presynaptic in origin because the postsynaptic chemosensitivity of the myasthenic muscle fibres was found to be normal¹, but recent evidence indicates that myasthenia gravis is an autoimmune disease directed against the postsynaptic acetylcholine receptors^{3,4}. Myasthenic serum globulin has been shown to bind to the acetylcholine receptors^{5,6}, myasthenic muscle contains only 11–30% of α -bungarotoxin binding sites compared with normal muscle⁷, and iontophoretic studies showed that the acetylcholine sensitivity of the postsynaptic region of myasthenic muscle was markedly reduced². But the failure of *in vitro* application of myasthenic serum to reduce the junctional or extrajunctional sensitivity of human, rat or frog muscle⁸ has raised doubt on the involvement of the

serum factor in the disease. In this report we show that the acetylcholine sensitivity of cultured rat myotubes is greatly reduced by *in vitro* application of myasthenic serum.

Skeletal muscle cells from newborn rats were explanted on collagen-coated 60 mm plastic Petri dishes according to the techniques of Yaffe⁹, at a plating density of 5×10^6 cells per dish. The cells were incubated in Dulbecco's modified Eagle's medium with 8 g l⁻¹ glucose, 9% foetal calf serum, 0.5% chick embryo extract and 50 μ g ml⁻¹ gentamycin. The cultures were maintained in a humidified atmosphere at 37 °C with 95% air and 5% CO₂. The myogenic cells began to fuse by day three and experiments were performed on 5 to 6 d old myotube cultures. Pooled sera from two patients with myasthenia gravis with a high titre of acetylcholine receptor antibody, and pooled sera from two normal individuals, were used. The normal or myasthenic serum was added to the 5–6 d old cultures containing the culture medium so as to give a sevenfold dilution of the serum.

Intracellular recordings were made using 3 M KCl-filled microelectrodes of 20–40 M Ω resistance, and the acetylcholine sensitivity measured iontophoretically using microelectrodes filled with 2 M acetylcholine and with a resistance of 50–200 M Ω . Recordings were made at 23 °C in the culture media except where otherwise stated. Acetylcholine receptors are located over the entire surface of cultured rat myotubes¹⁰, and the acetylcholine sensitivity was measured at several points on each myotube and the average sensitivity calculated. The sensitivity was expressed in mV nC⁻¹ and corrected for variation in membrane potential by multiplying the observed sensitivity by a factor $(E_s - E_R)/(E_M - E_R)$ where E_s is the standard membrane potential (–60 mV), E_R is the reversal potential for the acetylcholine response (0 mV) and E_M is the resting potential of the cell. The acetylcholine sensitivity was always measured for a depolarisation of 5 mV. It is particularly important to measure the acetylcholine sensitivity using a constant depolarisation when the sensitivity of different cells is being compared, since the sensitivity becomes higher with increasing depolarisation if the dose–response curve is nonlinear. We have found that the action of acetylcholine on rat myotubes does show such cooperativity, with a Hill coefficient of 1.5–2.0.

The acetylcholine sensitivity of the myotubes was initially measured in three separate sets of cultures with half of each set of cultures incubated for 22.5 h in normal serum, and the remainder incubated for the same period in myasthenic serum (Table 1). The myasthenic serum did not alter the resting potential of the myotubes, E_M of the normal and myasthenic cells averaging –47 mV and –45 mV respectively. The acetylcholine sensitivity of the cells treated with myasthenic serum was greatly reduced, however, in all of the three sets of cultures. The average acetylcholine sensitivity of the normal myotubes was 399 mV nC⁻¹, while that of the myasthenic myotubes was 27 mV nC⁻¹. Thus 22.5 h incubation in myasthenic serum caused a 93% reduction in the acetylcholine sensitivity.

Table 1 Reduction of acetylcholine (ACh) sensitivity of cultured rat myotubes by myasthenia gravis serum

Culture	No. of cells	Normal ACh		Myasthenia ACh		Inhibition of ACh sensitivity in myasthenic cells (%)
		E_M (mV)	sensitivity (mV nC ⁻¹)	E_M (mV)	sensitivity (mV nC ⁻¹)	
1	6	32	175	36	12	93*
2	5	50	825	46	52	94*
3	6	55	198	58	16	92*

The myotubes were incubated in normal serum or myasthenic serum for 22 h. Statistical significance was determined using a t test. E_M , membrane potential.

* $P < 0.001$.

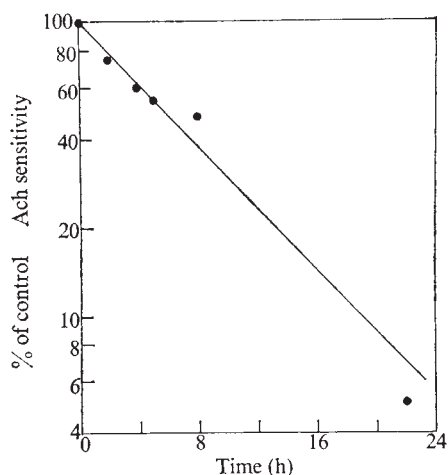


Fig. 1 Acetylcholine sensitivity of cultured rat myotubes incubated for various times in myasthenic serum. Cultures were incubated for the times shown in normal or myasthenic serum, and the average value of the ACh sensitivity of six to eight myotubes cultured in normal or myasthenic serum was compared for each incubation time.

This reduction in sensitivity was irreversible, as perfusing for 3 h in normal saline caused no increase in the acetylcholine sensitivity. The acetylcholine potentials were also much slower in the myotubes treated with myasthenic serum. Normal myotubes gave 5 mV amplitude acetylcholine potentials with 10–20-ms rise times, whereas myotubes incubated with myasthenic serum gave 5 mV potentials with only 50–100-ms rise times. The acetylcholine sensitivity varied slightly at different points on both normal and myasthenic serum treated myotubes, but we found no evidence of acetylcholine receptors accumulating in patches or in caps at one area of the myotubes.

The rate of the decrease in acetylcholine sensitivity was very slow, and 2 h after incubation with the myasthenic serum, the sensitivity had decreased by only 25%. Figure 1 shows the time course of reduction of acetylcholine sensitivity, plotted on a semi-logarithmic scale. The reduction in sensitivity is approximately exponential, with a decay rate constant of 0.124 h^{-1} and a half-time of 5.6 h.

These results demonstrate that the serum of myasthenic gravis patients contains a factor which can reduce the acetylcholine sensitivity of cultured rat myotubes. Previous biochemical studies have shown that this factor is an IgG globulin which can bind to the acetylcholine receptor^{5,6}, although the IgG binding site is different from the acetylcholine binding site^{6,8}.

The slow rate and irreversible nature of the reduction in acetylcholine could be explained by the serum globulin being an irreversible antagonist and present in very low concentrations. The globulin has been shown, however, to produce an acceleration of the internalisation and/or degradation of the surface acetylcholine receptors of the cultured rat myotubes¹¹. It therefore seems likely that the binding of the globulin to the receptor does not prevent activation of the receptor by acetylcholine, but the reduction in acetylcholine sensitivity is caused by a decrease in the density of surface receptors on the myotubes.

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Human myasthenic sera reduce acetylcholine sensitivity of human muscle cells in tissue culture

MYASTHENIA gravis is a muscle disease of man characterised by impaired neuromuscular transmission during repetitive motor nerve activity. Recent studies suggest that the defect in myasthenia gravis is post-synaptic and that myasthenic end-plates have a lower density of functional acetylcholine (ACh) receptors than normal end-plates. Thus end-plates from myasthenic patients show unusually small miniature end-plate potentials^{1,2}, are less sensitive than normal end-plates to iontophoretically applied ACh (refs 2, 3) and bind reduced amounts of α -bungarotoxin^{4,5}—a snake neurotoxin which is considered to bind specifically to nicotinic ACh receptors (see ref. 5). The presence of antibodies against ACh receptors in the sera of myasthenic but not of control patients^{8–10} suggests that anti-receptor antibodies might act in some way to decrease the number of functional receptors at the motor end-plate. Previous investigations, however, have failed to show conclusively that myasthenic sera contain a factor that reduces muscle ACh sensitivity^{3,11–13}. Here we show that myasthenic sera can reduce the ACh sensitivity of tissue cultured human muscle cells.

Explant cultures were made from muscle tissue of 12–16 week old foetuses. Cultures were grown at 37 °C on gelatin-coated tissue culture dishes in a modified Eagle's medium¹⁴ containing 10% foetal calf serum. Outgrowths of fibroblasts and myoblasts were seen after about 2 weeks and fused myotubes appeared after 3 weeks. The cultures selected for study were aged 4–7 weeks.

Iontophoretic application of ACh on to human myotubes evoked depolarising responses (Fig. 1). To compare ACh responses in cells of different resting potentials, ACh sensitivities were corrected to a resting potential of -50 mV by multiplying the recorded sensitivities by $-50/RP$, where RP is the resting potential (mV) of the cell under study. This correction factor assumes that the reversal potential for ACh responses is 0 mV (our unpublished observations). Cultures pre-incubated with 10% normal human serum at 37 °C for 1.5 h were used to provide a control value for ACh sensitivity. The mean sensitivity of myotubes exposed to three different normal sera was $1,080 \text{ mV nC}^{-1}$ (range of mean values, $800\text{--}1,200 \text{ mV nC}^{-1}$), which is similar to that of untreated cultures (about 950 mV nC^{-1}).

Six myasthenic sera were tested for their effect on myotube ACh sensitivity. Cultures were incubated with 10% serum at 37 °C for 1–1.5 h before electrophysiological examinations in serum free solution. As shown in Table 1, all the sera reduced myotube ACh sensitivity; in some cases the reduction was surprisingly large. This lowering of sensitivity is complement independent since a similar decrease was seen for one serum (no. 6 in Table 1) when complement was inactivated by heating the serum to 56 °C for 30 min. Myotube electrical properties which can influence the measured ACh sensitivity were similar in cultures exposed to either normal or myasthenic sera (Table 2). The lower sensitivity of myotubes treated with myasthenic sera must, therefore, result from an action of the serum on ACh receptors. Two sera from patients with

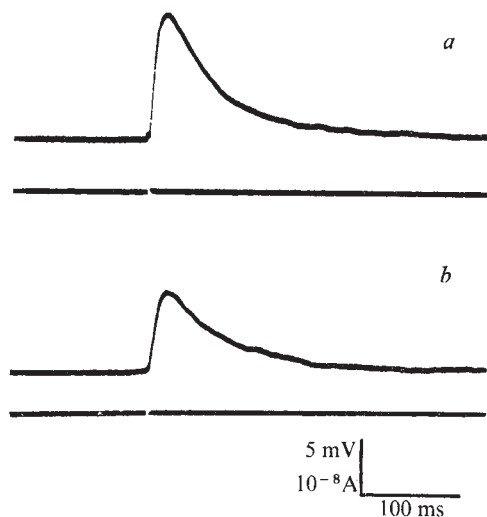


Fig. 1 Depolarising responses evoked by iontophoretic ACh pulses of 1 ms duration in myotubes treated with *a*, normal human serum and *b*, myasthenic human serum (serum 2 in Table 1). Recordings were made at 23 °C in a serum-free solution containing (mM) NaCl 150, KCl 5, CaCl₂ 10, HEPES-NaOH 5, pH 7.4. ACh was applied iontophoretically from pipettes filled with 3 M AChCl (resistances 180–400 MΩ using pulses of 5–20 nA amplitude and 0.5–10 ms duration). The position of the ACh pipette and the negative backing current required to prevent ACh diffusing out of the pipette were adjusted for each myotube to ensure a maximal ACh response. The resultant myotube depolarisations were recorded with an intracellular microelectrode filled with 4 M potassium acetate (resistances 20–40 MΩ). Recording pipettes were bevelled²⁷ to facilitate penetration of the small myotubes. ACh sensitivities were calculated as mV depolarisation per nanocoulomb (nC) of charge passed through the iontophoretic pipette.

Duchenne muscular dystrophy and amyotrophic lateral sclerosis were also tested; these had little or no effect on ACh sensitivity.

An immunoglobulin (IgG) fraction of myasthenic sera has been shown to block α -bungarotoxin binding to ACh receptors^{6,7}. To test if IgG is also involved in reducing ACh sensitivity, one serum was treated with goat anti-human IgG to precipitate IgG. The IgG-depleted serum showed a greatly reduced activity whereas serum treated with normal goat serum retained its full potency (see Table 1).

The effect of incubating cultures with myasthenic sera at a lower temperature was tested in four cases. Cultures were incubated with 10% serum for 1.5 h at 23 °C before estimating ACh sensitivities. All four sera reduced myotube sensitivity, though the decrease in each case was less than when cultures were exposed to myasthenic serum at 37 °C (Table 1). In two of these experiments, the cultures were subsequently incubated in serum-free medium at 37 °C for an additional 1 h. This resulted in a further decrease in sensitivity; the relative sensitivity of cells incubated with sera 3 and 6 fell from 0.41 and 0.47, respectively, to 0.18 in both cases. From this result we conclude that the temperature dependence of the serum effect is not due to different amounts of antibody binding at the two temperatures, but rather reflects a subsequent step in antibody action.

Experimentally raised anti-receptor antibodies increase the rate of receptor degradation in cultured rodent muscle cells¹⁵. This degradation, which resembles antigenic modulation^{16,17}, is temperature dependent and is blocked by metabolic inhibitors. The similar temperature dependence of human myasthenic serum action suggests that antibodies in the sera act to raise the receptor degradation rate in human myotubes. Indeed when five of the myasthenic sera were tested on cultured mouse muscle cells, all were found to increase the rate of ACh receptor degradation (Fig. 2). Receptor degradation follows first-order kinetics^{18–21},

Table 1 Effect of human sera on the ACh sensitivity of human myotubes

Serum	Anti-human ACh receptor titre ($\times 10^{-9}$ M)	Relative ACh sensitivity	
		23 °C	37 °C
Normal	<0.6	1.00	1.00
Myasthenic patient no.			
1	30	0.46	0.02
2	8	—	0.39
3	446	0.69, 0.41	0.20
4	332	0.71	0.40
5	143	—	0.53
6	83	0.47	0.03
6, complement inactivated	—	—	0.06
6, IgG precipitated with goat anti-human IgG	—	—	0.65
6, plus normal goat serum	—	—	0.08
Duchenne muscular dystrophy	<0.6	—	0.95
Amyotrophic lateral sclerosis	<0.6	—	1.19

Relative ACh sensitivities of myotubes after exposure to 10% human sera at either 23 or 37 °C for 1–1.5 h. Sensitivities are expressed as the ratio (test serum ACh sensitivity–normal serum ACh sensitivity). For each serum sensitivities were estimated in 6–20 cells. The unity value for normal serum represents the mean sensitivity of six experiments testing three sera. Anti-human ACh receptor titres were estimated using an immunoprecipitation technique described elsewhere²⁸. Titres are expressed as moles of ¹²⁵I- α -bungarotoxin bound to human ACh receptors precipitated per litre of serum. In one experiment, serum 6 was depleted of IgG by precipitation with goat anti-human IgG serum; complement was inactivated in both sera. The resultant supernatant was dialysed against culture medium for 24 h before adjusting the volume to give the equivalent of 10% human serum in medium. Some reduction in sensitivity was seen when this IgG-depleted serum was tested. It seems likely that this is due, at least in part, to the lower input impedances of the myotubes in this experiment (about 40% of other values). To test for any general effects of goat serum on myasthenic serum action, the immunoprecipitation protocol was repeated using normal goat serum in place of immune goat serum. In this case no precipitate was formed and the full reduction in ACh sensitivity was seen.

whereas the appearance of new receptors is a linear process^{19,21,22}. An antibody-induced acceleration of receptor degradation without a compensatory increase in the rate of synthesis would therefore result in a lowered receptor density; this could explain the reduced ACh sensitivity of cells exposed to myasthenic sera. A possible objection to this idea is the observation that while the ACh sensitivity of myasthenic serum-treated cells declines within 60–90 min, the accelerated secretion of degraded ¹²⁵I- α -bungarotoxin, which is used to assay receptor degradation, occurs after a lag of several hours (Fig. 2 and ref. 15). This lag, however, includes the time not only for induced receptor internalisation but also for receptor catabolism and secretion of the degraded toxin. The internalisation of antibody-complexed cell surface components can occur within 1 h (ref. 23) and the secretion of degraded ¹²⁵I- α -bungarotoxin occurs in

Table 2 Myotube electrical properties after exposure to human serum at 37 °C

	Resting potential (mV)	Input impedance (MΩ)	Membrane time constant (ms)
Normal sera	-47.9 $\pm$ 4.4	244 $\pm$ 76	71 $\pm$ 17
Myasthenic sera	-47.7 $\pm$ 3.7	225 $\pm$ 28	77 $\pm$ 8

Values are expressed as mean $\pm$ s.e. of between experiment results. The measured resting potentials represent the potentials at which ACh sensitivities were measured and probably underestimate the true value. Cell input impedances and membrane time constants were measured by passing hyperpolarising pulses of 0.2 nA amplitude and up to 1 s duration through the recording electrode using a bridge circuit built into the electrometer (M-4A, W.P. Instruments). As the time constant of the myotube membrane was much greater than that of the recording system, the bridge circuit was easily balanced with the electrode inside the cell. The large input impedances reflect the small size of the cells (5–20 μ m diameter) rather than any unique membrane properties.

normal conditions with a lag of about 1 h (ref. 19). An antibody-induced modulation of ACh receptors may, therefore, lower the surface receptor density within the 1–1.5 h incubation periods used in the electrophysiological experiments.

Bound antibodies could also reduce ACh sensitivity by modifying the properties of activated ACh receptors. Preliminary 'ACh noise' experiments indicate that in human myotubes exposed to myasthenic serum the charge passed during a single channel opening is reduced by about 30% (S.B., R.W.K. & J. Rice, unpublished). A similar reduction in the charge transferred per open channel is seen in cultured rat myotubes treated with experimentally raised antibodies¹⁵. In addition, antibodies might act by completely inactivating some ACh receptors. Such direct actions of antibodies on receptor function may explain the lowered ACh sensitivity of cells exposed to myasthenic serum at 23 °C and also contribute to the larger reduction seen with incubations at 37 °C.

Our demonstration that myasthenic sera can reduce muscle ACh sensitivity contrasts with the results of earlier studies. As anti-receptor antibodies show a higher affinity for homologous than for heterologous receptors^{24,25} the lack of any serum effect in some previous studies may be due to the use of non-human muscle cells. In addition, more labile receptors such as those in tissue cultured cells^{19,21} may be necessary to show a rapid reduction in sensitivity arising

from receptor modulation. The loss of the more stable end-plate receptors^{18,20,26} through modulation may occur too slowly to cause any decrease in ACh sensitivity during short term experiments. This would explain the apparent absence of any blocking action of myasthenic sera at human end-plates *in vitro*³.

Our results suggest that the low ACh sensitivity of human myasthenic end-plates results primarily from the action of antibodies. Further experiments will be necessary to determine if myasthenic sera do decrease human end-plate sensitivity in appropriate conditions and to elucidate further the importance of receptor modulation in depressing ACh sensitivity.

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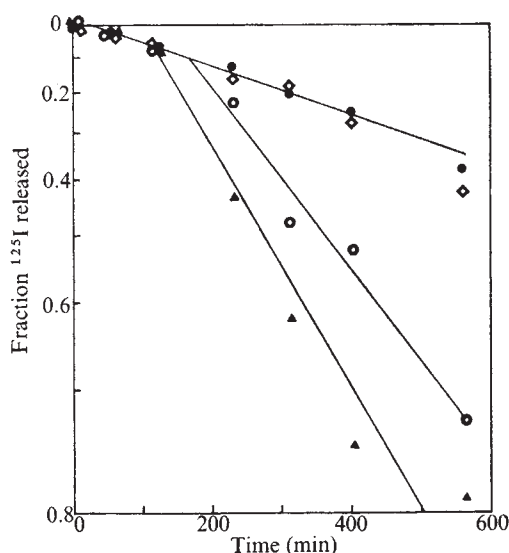


Fig. 2 Loss of ¹²⁵I-α-bungarotoxin from mouse muscle cells. Cells of the clonal cell line BC₃H-1 (refs 15, 21) were incubated in 10⁻⁸ M ¹²⁵I-α-bungarotoxin for 2 h at 21–23 °C in medium containing 0.2% foetal calf serum. Unbound toxin was then removed by washing the cells four times in medium with 0.2% foetal calf serum. The cultures were then incubated at 37 °C in medium containing various sera and the amount of ¹²⁵I released into the medium measured at various times. ¹²⁵I release is expressed as the ratio ¹²⁵I released/amount of ¹²⁵I initially bound to cells. Each point is the mean of duplicate samples. Fractionation of the medium on a Biogel P2 column showed that the extra ¹²⁵I released in the presence of myasthenic serum is due to an increase in the release of low molecular weight ¹²⁵I-labelled material and not to an induced dissociation of intact bungarotoxin. ◇, Foetal calf serum (FCS, 12%); ●, 10% normal human serum + 2% FCS; ○, 10% myasthenic human serum + 2% FCS. For comparison the effect on these cells of serum from rats immunised with electric eel ACh receptor is also shown (▲, 10% rat serum + 2% FCS, final titre 1.1 × 10⁻⁸ M anti-rat receptor). In a further experiment 10% human serum was added to cultures in the presence of 10⁻⁸ M ¹²⁵I-α-bungarotoxin and 10⁻³ M curare. In these conditions toxin binding to the muscle cells is inhibited by more than 90%. After 600 min the medium was removed and fractionated. No degradation of the ¹²⁵I-α-bungarotoxin was noted, indicating that the myasthenic serum, by itself, does not degrade toxin and that toxin degradation only occurs after binding to ACh receptors.

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Long term β-adrenergic blockade reduces tyrosine hydroxylase and dopamine β-hydroxylase activities in sympathetic ganglia

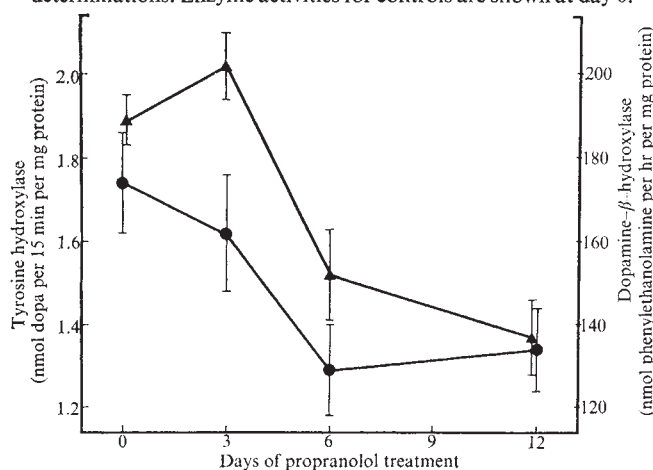
β-ADRENOCEPTOR antagonists are now well established in the treatment of hypertension but the mechanism by which they reduce blood pressure is not understood¹. There is clearly more involved than an immediate blockade of the cardiovascular β-adrenergic receptors, since intravenous (i.v.) infusion of propranolol in man reduces cardiac output but has no effect on arterial blood pressure^{2,3}. The full hypotensive effect of β-adrenergic blockers is delayed⁴, and is associated with a reduction in peripheral resistance^{2,3}. The hypotensive action of these drugs is nevertheless predominantly related to their common property of β-adrenergic receptor blockade, as they all produce similar

falls in blood pressure⁵ despite their differing ancillary properties⁶ and effects on plasma renin activity⁷. It has been suggested that the hypotensive effect of propranolol is due to a central inhibition of sympathetic activity, since i.v. propranolol reduces preganglionic sympathetic nerve activity in rabbits⁸. But practolol, an effective antihypertensive which penetrates the central nervous system poorly⁹, has no such acute effect¹⁰. The possibility has yet to be considered that the hypotensive action common to all β -adrenoceptor antagonists is directly related to a slowly developing reduction in sympathetic activity and hence in peripheral resistance. Long term increases or decreases in the activity of sympathetic neurons are specifically reflected by changes in the concentration of the nor-adrenaline-synthesising enzymes tyrosine hydroxylase and dopamine β -hydroxylase contained in the cells¹¹. We report here that prolonged treatment with β -adrenoceptor antagonists causes a slow-onset reduction in the concentration of these enzymes in the superior cervical ganglia of rabbits. This decrease in sympathetic activity may in part explain the hypotensive effect of long-term β -blockade.

Rabbits (2–3 kg) were injected twice daily subcutaneously (s.c.) with ($\pm$)-propranolol for 3, 6 or 12 d, or with saline. The degree and duration of β -blockade achieved with this dosage regime have been previously reported¹². In further experiments animals were dosed for 24 d with either ($\pm$)-propranolol, (+)-propranolol (ICI), ($\pm$)-metoprolol, ($\pm$)-acebutolol, ($\pm$)-practolol in equipotent dosage or with saline. Animals were stunned 18–24 h after the final injection and the left and right superior cervical ganglia were rapidly removed and homogenised separately in 1 ml of 0.3 M sucrose. Tyrosine hydroxylase activity of homogenates was measured by the method of Levitt *et al.*¹³ and dopamine β -hydroxylase activity by that of Molinoff *et al.*¹⁴. Protein was determined by the method of Lowry *et al.*¹⁵. To compare the effect of decentralisation on ganglionic enzyme activity with that of prolonged β -adrenoceptor blockade, the left superior cervical preganglionic trunk was cut and ligated under nembutal anaesthesia in four rabbits. After a recovery period of 12 d both superior cervical ganglia were removed and the activities of their enzymes were measured.

Propranolol has no acute effect on tyrosine hydroxylase activity in sympathetic ganglia¹⁶ and similarly we found no significant change in ganglionic tyrosine hydroxylase or dopamine β -hydroxylase activities after 3 d of propranolol treatment. In contrast, activities of both enzymes were reduced by pretreatment with propranolol for 6 d (Fig. 1),

Fig. 1 Effect of chronic treatment with ($\pm$)-propranolol on activities of tyrosine hydroxylase (●) and dopamine β -hydroxylase (▲) in rabbit superior cervical ganglia. Rabbits were injected s.c. twice daily with ($\pm$)-propranolol 4 mg per kg body weight for 3 d ($n = 4$), 6 d ($n = 4$) or 12 d ($n = 4$) or with saline 1 ml per kg ($n = 6$). Points are mean $\pm$ s.e.m. of 8–12 determinations. Enzyme activities for controls are shown at day 0.



and after 12 d tyrosine hydroxylase and dopamine β -hydroxylase activities were 25% lower than in controls. To determine whether this action was due to β -receptor blockade and not to any subsidiary properties of the drugs, rabbits were treated for 24 d with four β -adrenoceptor antagonists chosen for their differing properties of cardio-selectivity, intrinsic sympathomimetic activity and membrane-stabilising action⁶. Despite these differences all four drugs produced comparable reductions in enzyme activities (Table 1). The fall in dopamine β -hydroxylase activity was similar for all drugs, but the reduction in tyrosine hydroxylase activity was greatest with propranolol, and least with practolol. This cannot be due to the relatively powerful membrane-stabilising activity of propranolol since (+)-propranolol, which has negligible β -blocking activity but the same local anaesthetic potency as ($\pm$)-propranolol, did not alter enzyme activities (Table 1). The difference between propranolol and practolol may be related to the high lipid solubility and central nervous system (CNS) penetration of propranolol¹⁷ compared with that of practolol¹⁸.

Table 1 Effect of chronic treatment with β -adrenoceptor antagonists on tyrosine hydroxylase and dopamine β -hydroxylase activities in rabbit superior cervical ganglia

	Tyrosine hydroxylase (% of control)	Dopamine β -hydroxylase (% of control)
($\pm$)-Propranolol	64 $\pm$ 8*	70 $\pm$ 6*
(+)-Propranolol	91 $\pm$ 10	103 $\pm$ 8
($\pm$)-Metoprolol	70 $\pm$ 7*	73 $\pm$ 6*
($\pm$)-Acebutolol	79 $\pm$ 7†	64 $\pm$ 13†
($\pm$)-Practolol	85 $\pm$ 5‡	73 $\pm$ 5*

Rabbits were injected s.c. twice daily for 24 d with either saline 1 ml per kg ($\pm$)-propranolol 4 mg per kg, (+)-propranolol 4 mg per kg, ($\pm$)-metoprolol 6 mg per kg, ($\pm$)-acebutolol 10 mg per kg or ($\pm$)-practolol 10 mg per kg body weight. Enzyme activity is expressed in terms of nmol dopa per 15 min per mg protein (tyrosine hydroxylase) or nmol phenylethanolamine per h per mg protein (dopamine β -hydroxylase) given as percentage of control activity. Values are mean $\pm$ s.e.m. of 10–12 determinations.

* $P < 0.001$.

† $P < 0.02$.

‡ $P < 0.05$.

The effect of decentralisation of superior cervical ganglia is shown in Table 2. Twelve days after decentralisation, activities of tyrosine hydroxylase and dopamine β -hydroxylase were 26% and 29% less than in contralateral control ganglia, a reduction comparable with that caused by prolonged β -adrenergic blockade.

Adaptation to increases in sympathetic activity by trans-synaptic induction of tyrosine hydroxylase and dopamine β -hydroxylase is due to increased synthesis of enzyme protein¹¹. This process is slow, and first detectable 18 h after increased preganglionic activity¹⁸. Conversely it is thus possible that β -adrenoceptor antagonists cause a sudden reduction in preganglionic nerve activity, which is detected in these experiments as a reduced enzyme activity in the sympathetic neurons. As we observed no significant change in enzyme activities after 3 d of propranolol treatment, however, it is more probable that continuing β -adrenergic blockade causes a gradual reduction in preganglionic sympathetic activity mirrored by the reduction in the activities of the specific neuronal enzymes. Strikingly, we found that interruption of preganglionic impulse flow by decentralisation resulted in a very similar fall in the enzyme activities in the superior cervical ganglia. A possible interpretation of this is that chronic β -adrenergic blockade is virtually abolishing all preganglionic nervous activity. Of course, we cannot know if this is so because it is not known by how much the nervous activity must be reduced to detect the consequent decrease in enzymic activity. If preganglionic

Table 2 Effect of decentralisation on the tyrosine hydroxylase and dopamine β -hydroxylase activities in rabbit superior cervical ganglia

	Tyrosine hydroxylase (nmol dopa per 15 min per mg protein)	Dopamine β -hydroxylase (nmol phenylethanolamine per h per mg protein)
Control (R)	2.02 $\pm$ 0.25	209 $\pm$ 9
Decentralised (L)	1.47 $\pm$ 0.18*	149 $\pm$ 16*

Left superior cervical ganglia were decentralised in four rabbits, and 12 d later the activities of the enzymes were determined. Values given are the mean $\pm$ s.e.m. of four ganglia. To ensure that the ganglia were decentralised choline acetyltransferase activity was determined in the homogenates according to the method of Fonnum²⁰. The activity of this enzyme in the decentralised ganglia was less than 10% of the contralateral controls.

* $P < 0.01$ (paired t test).

activity is reduced by blockade of central β -adrenoceptors⁸ our results would suggest that polar 'cardioselective' drugs may reach significant concentrations in the CNS during steady-state dosing. Alternatively, the reduction in sympathetic activity by chronic β -adrenergic blockade may be peripherally mediated either through a long term alteration of afferent autonomic input, resulting in an adaptive reduction in sympathetic outflow^{1,10}, or through continued blockade of a prejunctional β_1 -adrenoceptor, activation of which increases noradrenaline release¹⁹.

It seems reasonable, though, to conclude that prolonged β -adrenergic blockade produces a slow-onset reduction in the activity of the sympathetic nervous system. Since tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of noradrenaline²¹, the amount of neurotransmitter available for release is most probably also reduced. Significant reductions in the ganglionic activities of tyrosine hydroxylase and dopamine β -hydroxylase were seen with several different β -adrenoceptor antagonists, all of which are clinically effective antihypertensives despite their differing subsidiary properties. We therefore suggest that this delayed reduction in sympathetic activity contributes to the hypotensive action of β -adrenoceptor antagonists.

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β Endorphin inhibits ACh turnover in nuclei of rat brain

NARCOTIC drugs modify cholinergic mechanisms in central and peripheral nervous systems¹, and analgesic doses of morphine, meperidine, viminol R₂ and azidomorphine decrease acetylcholine (ACh) turnover rate (TR_{ACh}) in selected brain nuclei^{2,3}. This suggests that endogenous opiate receptor agonists regulate cholinergic mechanisms in some brain nuclei². Among the endogenous opiate receptor agonists, β endorphin is particularly interesting because it is more active than morphine in causing analgesia and catalepsy⁴. This polypeptide and α endorphin share some structural similarities which correspond to fragments 61-76 and 61-91 of β lipotropin, respectively⁵. We have investigated whether α and β endorphin also mimic the action of morphine on TR_{ACh} of selected brain nuclei. We have found that analgesic doses of β endorphin injected intraventricularly decrease TR_{ACh} in cortex, hippocampus, nucleus accumbens and globus pallidus but not in nucleus caudatus. This decrease of TR_{ACh} was antagonised by naltrexone. In contrast, α endorphin failed to cause analgesia or to decrease TR_{ACh} in all brain nuclei studied.

Endorphins were injected into unanaesthetised rats (150 g male Sprague-Dawley, Zivic Miller, Allison Park, Pennsylvania) through polyethylene cannulae permanently implanted in the lateral ventricles, 2 d before the experiments. Each polypeptide was dissolved immediately before use in 10 μ l of saline. The analgesic activity of α and β endorphins was measured by the tail flick method⁷ 30 min after intraventricular injection.

TR_{ACh} was measured by intravenous infusion of phosphorylcholine (ammonium salt) (15 μ mol min⁻¹) for 9 min beginning 21 min after injection of endorphins. During the administration of phosphorylcholine rats were restrained in plastic cages. TR_{ACh} was calculated from the deuterium enrichment of choline (Ch) and ACh as before⁸. Rats were killed with a high-intensity microwave beam focused on the head⁹. Brains were removed and sliced into 400- μ m thick coronal sections and punches were made in the head of the nucleus caudatus, in the pars dorsalis of the hippocampus, in the globus pallidus, nucleus accumbens and parieto-occipital cortex. The position of the polyethylene cannulae was controlled during slicing.

The enrichment in deuterium of ACh and Ch extracted from various nuclei was measured mass fragmentographically⁸. From these values we calculated the fractional rate constant for the efflux of deuterated Ch and ACh. This value multiplied by the ACh content of each brain region gave TR_{ACh} (ref. 10). Table 1 shows that β endorphin inhibited TR_{ACh} in the hippocampus, nucleus accumbens, globus pallidus and cortex. But, like morphine, this polypeptide failed to change TR_{ACh} in the nucleus caudatus. From the data of Table 2, the dose of β endorphin that, given intraventricularly, reduced TR_{ACh} in hippocampus by 50%, was calculated to be approximately 0.2 nmol. From previous reports^{2,3}, the dose of morphine that caused similar changes in TR_{ACh} in the hippocampus was about 33 nmol g⁻¹, given intraperitoneally. Even allowing for differences due to the different route of drug administration, there is no doubt that β endorphin is several times more active than morphine in decreasing the TR_{ACh} of the hippocampus. Using the tail flick test, we have confirmed⁴ that β endorphin has analgesic activity (Table 2): 1.8 nmol per brain caused 50% of maximal analgesia⁷. Similar analgesic activity was obtained with morphine (35 nmol g⁻¹)². Thus, as shown for morphine, doses of β endorphin which elicited analgesia also reduced hippocampal TR_{ACh}. Naltrexone (14 μ mol kg⁻¹, given intraperitoneally) completely prevented the analgesic action and

Table 1 Effects of α and β endorphins on TR_{ACD} in nuclei of rat brain

Brain nuclei	Saline	β Endorphin (2.9 nmol per brain)	β Endorphin + naltrexone	α Endorphin (11.5 nmol per brain)
Cortex	1.0 $\pm$ 0.10	0.35 $\pm$ 0.059*	1.1 $\pm$ 0.14	0.97 $\pm$ 0.14
Hippocampus	3.9 $\pm$ 0.70	0.65 $\pm$ 0.099*	4.9 $\pm$ 2.0	3.7 $\pm$ 0.64
Globus pallidus	2.6 $\pm$ 0.40	1.0 $\pm$ 0.18*	2.9 $\pm$ 0.65	2.13 $\pm$ 0.19
Nucleus accumbens	4.9 $\pm$ 1.0	2.02 $\pm$ 0.35*	4.7 $\pm$ 1.0	4.1 $\pm$ 0.47
Nucleus caudatus	8.6 $\pm$ 0.80	8.4 $\pm$ 1.6	9.9 $\pm$ 1.6	6.7 $\pm$ 2.0

Data expressed in nmol per mg protein per h.

Endorphins were injected intraventricularly 21 min before intravenous infusion of phosphoryl (d_9) choline; naltrexone (14 μ mol per kg) was injected intraperitoneally 15 min before the endorphins. The ACh content in nmol per mg protein was 0.16 $\pm$ 0.03 in cortex, 0.30 $\pm$ 0.020 in hippocampus, 0.73 $\pm$ 0.060 in nucleus caudatus, 0.45 $\pm$ 0.034 in globus pallidus and 0.79 $\pm$ 0.045 in nucleus accumbens; the Ch content was 0.35 $\pm$ 0.030 in cortex, 0.33 $\pm$ 0.032 in hippocampus, 0.28 $\pm$ 0.020 in nucleus caudatus, 0.30 $\pm$ 0.020 in globus pallidus and 0.50 $\pm$ 0.035 in nucleus accumbens. The endorphins and naltrexone failed to change ACh and Ch content. Each value is the mean $\pm$ s.e.m. of 10 assays in the saline-treated group, and of five assays in the other groups.

* $P < 0.01$ compared with the saline-treated group.

the decrease of TR_{ACh} elicited by β endorphin. α Endorphin, in a dose of 11.5 nmol per brain, had no analgesic activity and failed to change TR_{ACh} in any of the five brain areas studied (Table 1). Thus β endorphin was biochemically and behaviourally more potent than α endorphin. This finding is in line with other reports showing the same order of potency in the displacement of 3H -etorphine from synaptosomal preparations of rat brain¹¹ or in behavioural effects⁴.

Table 2 Analgesia and TR_{ACh} in rat hippocampus after various doses of β endorphin

Dose of β endorphin (nmol per brain)	TR_{ACh} (nmol per mg protein per h)	Analgesia (% of maximum)
0 (saline)	4.1 $\pm$ 0.90	0
0.029	3.8 $\pm$ 0.70	7 $\pm$ 1
0.29	1.0 $\pm$ 0.15*	23 $\pm$ 5
1.45	—	36 $\pm$ 5
2.9	0.65 $\pm$ 0.099*	75 $\pm$ 15

Each value represents mean $\pm$ s.e.m. of 10 assays in saline-treated rats and five assays in endorphin-treated rats. Analgesia was evaluated by the tail flick test⁷.

* $P < 0.01$.

β Endorphin is an active, naturally occurring opiate receptor agonist, which, like morphine, decreases TR_{ACh} in doses which increase the threshold for pain sensation. The data reported here imply that if endogenous β endorphin were to be released from presynaptic terminals in the vicinity of opiate receptors located in the membrane of cholinergic neurones, it would decrease the metabolic rate of ACh and presumably decrease the rate of firing of these neurones.

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Calcium-dependent presynaptic action of substance P at the frog neuromuscular junction

SUBSTANCE P, an 11 amino acid peptide found in many areas of the nervous system¹, has been proposed to be the excitatory transmitter between primary afferents and motoneurons². A depolarising action of substance P on spinal motoneurons and primary afferents has been demonstrated^{3–6}. But, an initial reduction of response preceding an increased spinal reflex discharge and a reduction of the motoneurone excitatory postsynaptic potential has also been seen in response to substance P (refs 4, 5). In addition, both excitatory and inhibitory effects of substance P on firing of cuneate and cortical Betz cells have been reported^{7,8}. In the hatchetfish Mauthner fibre–giant fibre synapse, substance P has a pronounced presynaptic effect⁹. We have further investigated the effects of substance P at a synapse in which the electrophysiological characteristics and transmitter are known, the ionic constituents of the Ringer can be manipulated and quantal analysis is possible—the neuromuscular junction of the frog. We report here that substance P has a potent presynaptic effect on transmission at this synapse which is composed of an initial phase in which transmission is depressed, followed by a prolonged period in which excitability and quantal release are increased.

The cutaneous pectoris muscle and nerve of the frog, *Rana pipiens*, was used. With the exception of experiments (noted below) in which the Ca^{2+} concentration was varied, the Ringer contained 115 mM NaCl, 2 mM KCl, 0.6 mM CaCl and 5 mM MgCl buffered to pH 7.3 with Tes (Sigma). Substance P (Bachem) or eledoisin (eledoisin related peptide, Sigma) was added to the control Ringer, bath applied, and removed with repeated washing after 2–5 min exposure to the preparation. Substance P was used in the majority of the experiments; however, no difference in experimental results was seen when equivalent concentrations of eledoisin were used. Spontaneous miniature endplate potentials (m.e.p.p.) used for frequency and amplitude analysis were recorded from different experiments than those using evoked endplate potentials (e.p.ps). The e.p.ps were evoked by nerve stimulation at 1 per s for 1 min and stimulation discontinued between sampling periods. The m.e.p.ps used for quantal analysis were recorded before the evoked re-

sponse. Quantal content was calculated as e.p.p./m.e.p.p. amplitude. Control experiments of equal duration and stimulation pattern were run. Data analysis was as detailed previously⁹.

The effects of substance P on synaptic transmission were dose dependent and at high doses only depressant effects were seen. At 10^{-4} M concentration, m.e.p.p. amplitude and frequency, e.p.p. amplitude and quantal content were all reduced with the maximum depression being reached at 5–10 min after application. The m.e.p.p. amplitude usually showed a complete recovery but m.e.p.p. frequency, e.p.p. amplitude and quantal content seldom recovered to control values even 1–2 h after application. At lower peptide concentrations (10^{-5} M, 10^{-6} M), recovery of synaptic depression and a second phase of increased synaptic efficacy was seen. An initial depression of synaptic transmission was seen with a maximum depression at 5–10 min and a return to control values after 30–40 min followed by a prolonged second phase of increased synaptic efficacy in which the e.p.p. amplitude and quantal content showed increases of up to five times control values. This phase did not decline to control values until 2–3 h or more after peptide application. The m.e.p.p. amplitude showed little or no change at the lower peptide concentrations. The m.e.p.p. frequency changes usually showed a small frequency reduction in the initial phase and either no change or a small frequency reduction in the second phase. The latency of onset of the peptide effect was approximately 30 s at all peptide concentrations. No change in the resting potential of the muscle fibre was seen at any peptide concentration.

Figure 1 illustrates a typical response to application of 10^{-5} M substance P bath applied at the first arrow and removed at the second arrow. Both e.p.p. amplitude and quantal content reach a maximum reduction by 10 min,

Fig. 1 Representative example of the effect of substance P (10^{-5} M) on *a*, e.p.p. amplitude and *b*, quantal content. Substance P Ringer is applied to the bath at the first arrow and removed at the second. Data plotted as means and standard deviations at each indicated time period.

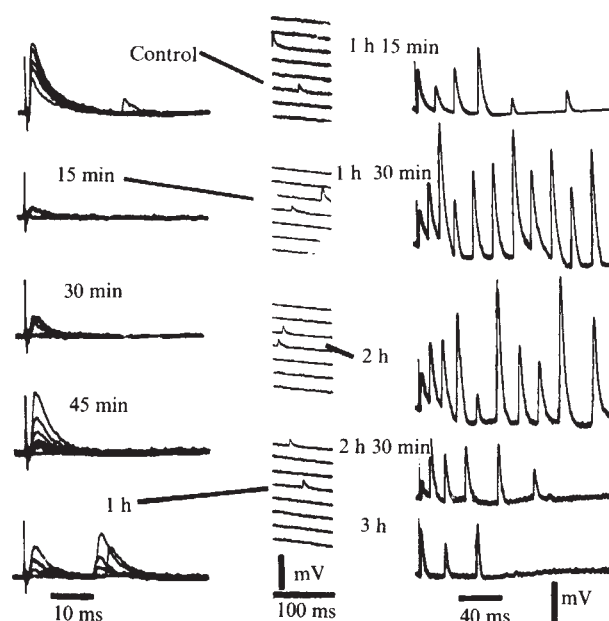
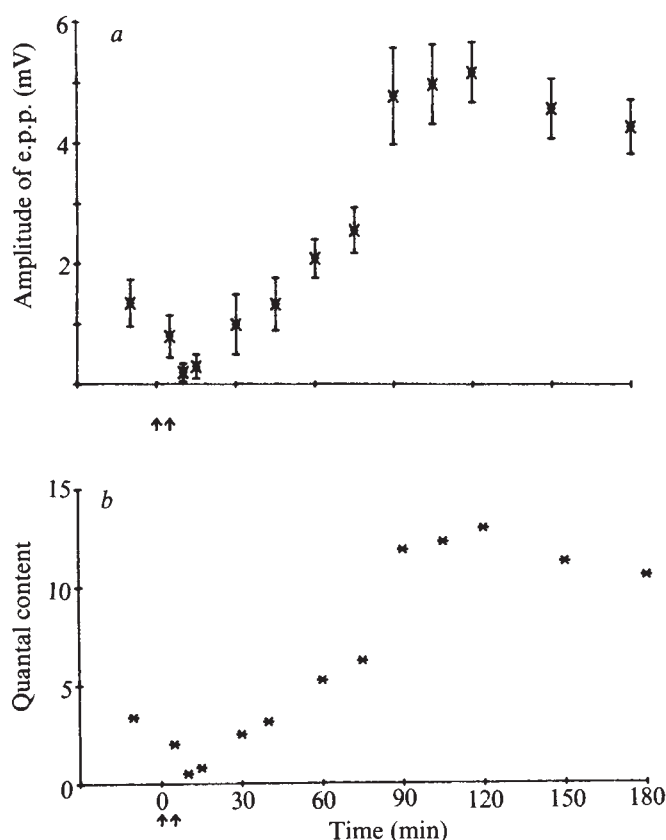


Fig. 2 Multiple e.p.p.s recorded from a muscle fibre following substance P application. Substance P (10^{-5} M) is applied after control record and removed by washing 5 min later. The time course of ensuing synaptic depression and recovery is the same as in Fig. 1 but on recovery, multiple e.p.p.s are produced in response to a single stimulus to the motor nerve. Figures in first column are composed of five consecutive sweeps; those in the last column are single sweeps. Note difference in time scale for first and last columns. Middle column contains samples from the same fibre of m.e.p.p.s at different time periods to illustrate lack of peptide effect on e.p.p. amplitude.

recover to control values at 40 min and then show a prolonged period of increase which, at 3 h post application when the cell was lost, was declining but had not yet returned to control values. In some experiments, during the phase of increased synaptic efficacy, multiple e.p.p.s were produced in response to a single stimulus to the presynaptic axon. This is presumed to be due to multiple firing of the motor axon. In Fig. 2, a maximum of 11 e.p.p.s are seen in response to a single stimulus to the motor axon. The multiple firing begins as the e.p.p. amplitude returns to control values and lasted, in this case, until the cell was lost 3 h after peptide application. The m.e.p.p. amplitude (middle column) shows no change during the entire experimental period. Although not apparent from the small m.e.p.p. samples in these records, m.e.p.p. frequency showed only a small reduction in frequency after peptide application, lasting for the duration of the experiment.

The initial phase of peptide-induced synaptic depression seems to be calcium dependent (Fig. 3). The reduction in e.p.p. amplitude produced by 10^{-4} M substance P can be blocked by increasing the Ca^{2+} in the Ringer. When the Ca^{2+} is increased in steps from 0.6 to 3.6 mM, an increase in e.p.p. amplitude with increase in Ca^{2+} is seen until at 3.6 mM Ca^{2+} no effect of the peptide on e.p.p. amplitude is seen: curare (1.5 mg l^{-1}) was added to the Ringer to prevent muscle twitching in response to nerve stimulation at high Ca^{2+} levels. In other experiments, in curare-free Ringer, no changes in m.e.p.p. frequency were seen when the peptide at 10^{-4} M was applied to the bath in Ringer containing 3.6 mM Ca^{2+} . The effect of increased calcium concentrations on the second phase of the response was not tested in these experiments. In addition to the above neurophysiological findings of a calcium dependency, a substance P-mediated reduction in ^{45}Ca uptake by isolated synaptosomes and an increase in ^{45}Ca sequestration by

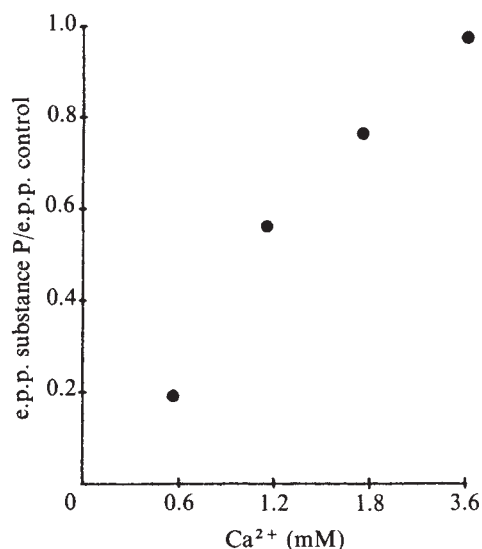


Fig. 3 Calcium dependence of the initial phase of synaptic depression produced by substance P. As calcium is increased, the reduction in e.p.p. amplitude produced by the peptide is decreased until, at twice normal Ringer calcium concentration (3.6 mM), the peptide has no effect on e.p.p. amplitude. Substance P concentration is 10^{-4} M. Each point is the average of five trials.

mitochondria isolated from rat brain has been found (unpublished data, A. Blitz, K. Dasse, and D. Sack). Effects on calcium uptake at these two sites both act to reduce free calcium in the presynaptic terminal. This, together with the above demonstrated blocking of the initial phase peptide effects by increased calcium in the Ringer, suggests that the synaptic depression seen in response to substance P is calcium dependent.

A possible mechanism of action for the substance P action reported here is a modulation of calcium-dependent potassium conductance which has been shown to regulate firing rates in several systems¹⁰⁻¹³. When delayed rectification is blocked in the frog neuromuscular junction, regenerative calcium dependent responses are seen in the presynaptic region¹⁴. A calcium-mediated potassium conductance has been suggested as a regulator of rhythmic and long term neural processes¹¹. That substance P may be a physiological means of regulating this process is consistent with the above data. One could then postulate that the peptide functions to bias the output produced by the more static short term information transfer of the conventional transmitters.

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Postsynaptic receptors are not essential for dopaminergic feedback regulation

ANTIPSYCHOTIC drugs (neuroleptics) which block central dopamine (DA) receptors increase the synthesis and metabolism of striatal DA and the firing of dopaminergic neurones in the substantia nigra. These effects have been postulated to be secondary to the drug-induced blockade of striatal postsynaptic DA-receptors and mediated by a negative feedback loop impinging on nigral DA-neurones^{1,2}. However, the existence of alternative mechanisms has been recently postulated³⁻⁵. In particular, it has been suggested that neuroleptics stimulate dopaminergic firing by blocking nigral DA-receptors, thus relieving DA-neurones from the inhibitory action of DA released from dopaminergic dendrites (self-inhibition)⁶. Indirect support for this possibility derives from the recent discovery of a DA-sensitive adenylate cyclase within the substantia nigra⁷⁻⁹. We report here that destruction of striatal DA-sensitive adenylate cyclase by kainic acid¹⁰ fails to reduce the effect of neuroleptics and of apomorphine, a DA-receptor agonist¹¹, on striatal DA-metabolism. These data indicate that an interaction with postsynaptic DA receptors is not essential for the changes in DA-metabolism produced by neuroleptics and apomorphine and provide *in vivo* evidence for the existence of a dopaminergic negative feedback independent from postsynaptic DA-receptors.

Male Sprague-Dawley rats (290-310 g) were anaesthetised with Equithesin and placed on a stereotaxic frame (Kopf); kainic acid (Sigma) dissolved in 1 μ l of saline was injected in 2 min in the caudate of one side while that of the other side received the same volume of saline. Coordinates were A 2.2, V 2.8, L 5.0 of Pellegrino and Cushman¹².

Ten days post-surgery, rats were killed and the striata of each side dissected out and homogenised in Tris-maleate buffer for adenylate cyclase assay¹³ or frozen at -20°C until analysed for DA and 3,4-dihydroxyphenyl acetic acid (DOPAC)¹⁴ in order to estimate DA-metabolism¹⁵. The brains of three rats were perfused with formaldehyde, cut by cryostat into 40- μ m slices and stained by cresylviolet in order to ascertain the extent and topography of the lesion.

Haloperidol (Janssen) and fluphenazine (hydrochloride salt, Squibb), were injected intraperitoneally (i.p.) while apomorphine, hydrochloride, Sandoz) was administered subcutaneously (s.c). Protein was measured by the method of Lowry *et al.*¹⁶ using bovine serum albumin as a standard.

Figure 1 is a micrograph of rat striatum from the side injected with kainic acid (3 μ g 10 d previously), as compared with the contralateral side injected with saline. It seems that in the side injected with kainic acid neuronal perikarya are markedly reduced in number while only a slight gliosis is present. The loss of neuronal perikarya affected more than 80% of the caudate-putamen, the nucleus interstitialis striae terminalis and about 60% of the nucleus accumbens, but did not involve the adjacent cerebral cortex and thalamus.

As soon as the rats awakened from anaesthesia they showed continuous turning contralateral to the kainic-injected side and intermittent whole body-rocking. This symptomatology lasted for at least 3-4 h. By 24 h post-surgery, rats displayed homolateral torsion of the body and turning which, although diminished, was still present 10 d post-surgery. At this time, administration of apomorphine (0.1 or 0.5 mg per kg body weight s.c.) stimulated the homolateral turning while haloperidol (0.3 mg per kg i.p.) reversed the turning into a contralateral one.

In agreement with the results of McGeer *et al.*¹⁰ we found that the unilateral intrastratial injection of 3 μ g of kainic acid resulted in a 40% decrease of basal adenylate cyclase activity in homogenates of kainic-injected striatum. Moreover, in these homogenates DA completely failed to stimulate the adenylate cyclase activity even when added at a concentration of 100 μ M, which maximally stimulated adenylate cyclase of striatal homogenates from the control side.

Unilateral administration of kainic acid did not modify DA-concentrations but resulted in a 100% increase of DOPAC levels in the lesioned striatum (see Fig. 2).

Parenteral administration of haloperidol increased, and of apomorphine decreased DOPAC levels both in the kainic-injected and in the control striatum. As Fig. 2 shows, 90 min after a maximally effective dose of haloperidol (0.3 mg per kg), DOPAC concentrations increased by the same amount in the kainic-injected and in the control striatum, thus reaching significantly higher levels in the lesioned striatum. A lower dose of haloperidol (0.100 mg per kg) caused a much greater accumulation of DOPAC in the kainic injected striatum than in the control side and the absolute increase of DOPAC in the kainic-injected side was more than twice that in the control one. Finally, a dose of haloperidol of 0.030 mg per kg failed to produce a significant increase of DOPAC in the control

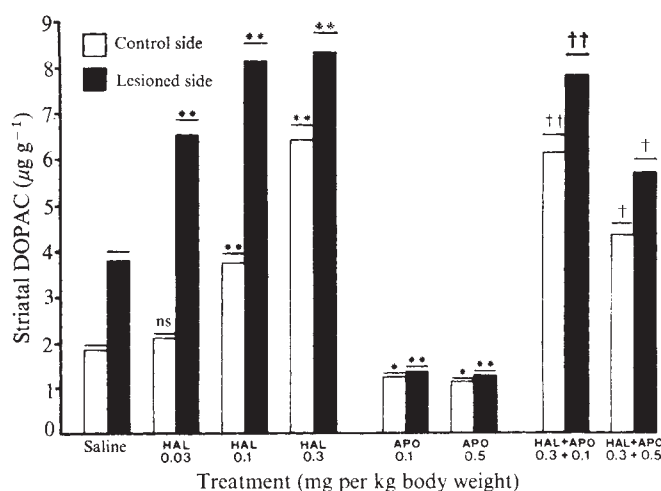
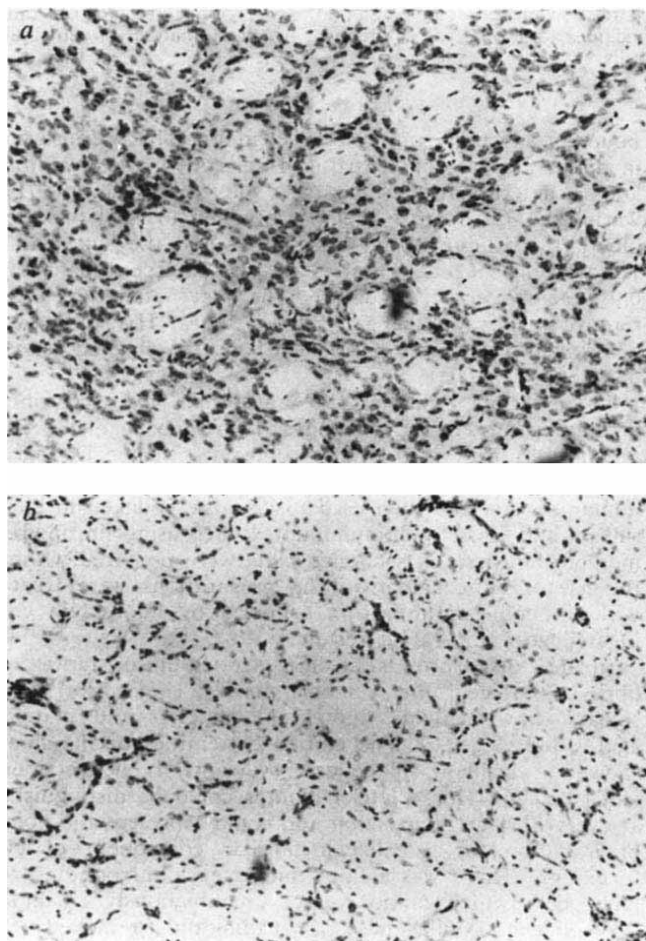


Fig. 2 Effect of haloperidol (H) and apomorphine (A) on DOPAC concentrations in control and kainic-lesioned striatum. Rats were injected with 3 μ g of kainic acid in the right striatum and with saline in the left one. After 10 d, rats were given haloperidol or apomorphine and were sacrificed 90 and 45 min respectively after drug administration. Each value is the mean $\pm$ s.e.m. of the number of determinations indicated in parentheses. * P < 0.01; ** P < 0.001, ns (not significantly different), in respect to the values of the homolateral striatum of rats administered with saline. † P < 0.01, ††, not significantly different, in respect to the values of the homolateral striatum of rats administered with haloperidol alone.

Fig. 1 Photomicrographs ($\times 5.25$) of a cresylviolet-stained section from a rat injected unilaterally in the striatum with kainic acid (3 μ g) 10 d earlier. *a*, Control striatum; *b*, kainic-injected striatum.



striatum but increased by about 100% the levels of DOPAC in the lesioned striatum. Similar results were obtained after 0.5, 0.2 and 0.05 mg per kg of fluphenazine.

Apomorphine administration (0.5 and 0.1 mg per kg; s.c.) produced a more pronounced decrease of DOPAC in the kainic-injected than in the control side. Finally, as shown in Fig. 2, apomorphine (0.1 mg per kg), failed to decrease DOPAC levels both in the kainic-injected and in the control striatum of rats pretreated with haloperidol (0.3 mg per kg). On the other hand, a higher dose of apomorphine (0.5 mg per kg) partly reversed the increase in DOPAC produced by haloperidol (0.3 mg per kg) in the lesioned and in the control striatum.

The complete loss of striatal DA-sensitive adenylate cyclase does not prevent the effect of DA-receptor blockers, as haloperidol and fluphenazine, and of a DA-receptor agonist, as apomorphine, on DA-metabolism. Since haloperidol and apomorphine were mutually antagonistic, their effects on DOPAC in the kainic-injected striatum should be mediated by an action on DA-receptors. Taking the loss of striatal DA-sensitive adenylate cyclase as demonstrating destruction of postsynaptic DA-receptors¹⁰, our results indicate that an action at the level of the postsynaptic DA-receptors is not a pre-requisite for the *in vivo* effects of DA-receptor blockers and agonists on DA-metabolism and strongly suggest the existence of an alternative dopaminergic mechanism exerting an inhibitory influence on DA-neurones.

The exact location of this mechanism remains to be established. It might be pre-junctional, being mediated by DA released from DA-terminals on to presynaptic DA-receptors regulating tyrosine hydroxylase^{15,16} and DA-release¹⁷ or located within the substantia nigra and mediated by DA released from dopaminergic dendrites on to DA-receptors localised on dopaminergic neurones⁶ or on connections afferent to the substantia nigra²¹.

A further interpretation of our data could be that the changes produced by the drugs in the kainic-injected striatum are mediated by an action on postsynaptic DA-receptors of non-striatal dopaminergic areas left intact by

the kainic treatment. This possibility is made unlikely, however, by the fact that the lesion produced by the intra-striatal injection of 3 μg of kainic acid was not restricted to the caudate-putamen but also involved other dopaminergic areas as the nucleus accumbens, the bed nucleus of the stria terminalis and the central nucleus of the amygdala. Prefrontal cortex was not found to be damaged but the paucity¹⁸ and uncertainty¹⁹ of the cortical afferences to the substantia nigra makes it difficult to admit that this area mediates the changes observed on the kainic-injected striatum. The tuberculum olfactorium was not affected by kainic acid but nigral afferences from this area have not been described¹⁸. Finally, the finding that the sensitivity of striatal DA-metabolism to neuroleptics and apomorphine is increased in spite of the large loss of brain DA-receptors produced by kainic acid is difficult to be interpreted as due to the activity of a residual extra-striatal negative feedback loop.

Note added in proof: A paper from Garcia-Munoz *et al.*²² has recently appeared, in which a different approach was used to reach the same conclusions of the present one.

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Hydroxyproline-containing protein adsorbed on to cellular elements of whole human blood

CURRENT hypotheses suggest that blood clotting occurs when, as a primary event, platelets come into direct contact with collagenous fibrils which have been exposed in vessel walls. We report here that a collagen component is normally associated with the cellular elements of blood. It was demonstrated by analysing whole blood and its fractions for protein-bound hydroxyproline, and shown by immunofluorescence in clotted blood smears using a specific antiserum to human collagen. As a blood component it is distinct from and additional to C1q, the hydroxyproline-containing protein which is a part of the first component of complement.

Hydroxyproline was first measured in serum and plasma by LeRoy¹ and was believed to be related to a soluble collagen component of blood. It was later found that this hydroxyproline was more probably related to C1q, which shares amino acid sequences including hydroxyproline, with collagen^{2,3}. The total serum concentration of hydroxyproline attributable to C1q, assuming a concentration of C1q in serum of 180 $\mu\text{g ml}^{-1}$ (ref. 4) and a hydroxyproline content of 5.4%, is 9.7 $\mu\text{g ml}^{-1}$ (ref. 5). Recent studies of whole citrated blood have shown about double this amount of hydroxyproline to be present, that is, 18 $\mu\text{g ml}^{-1}$ (ref. 6). The discrepancy between the serum and whole blood concentrations of hydroxyproline is apparently entirely due to collagen, which is all bound to blood cells.

Blood was drawn from three women (ages 25–52 yr) and three men (ages 23–67 yr) and hydroxyproline measured by the method of Woessner⁷ in whole blood, plasma and serum (Table 1). In agreement with the literature, plasma (or serum) has less than 10 $\mu\text{g ml}^{-1}$ bound hydroxyproline, so that the additional 10 $\mu\text{g ml}^{-1}$ originates from proteins

Table 1 Hydroxyproline levels in whole human blood and blood fractions

	Whole citrated blood ($\mu\text{g ml}^{-1}$)	Serum ($\mu\text{g ml}^{-1}$)	Plasma ($\mu\text{g ml}^{-1}$)	Plasma + cell washings* ($\mu\text{g ml}^{-1}$)	Cells† ($\mu\text{g ml}^{-1}$)
Means for six subjects	18.0	8.0	8.1	18.1	25.0
Range	15.4–23.3	6.8–10.5	7.7–11	15–20	21.2–38.8

*Additional and separate group of five subjects.

†Cell mean value calculated on the basis of haematocrit; cell range includes additional values obtained by direct analyses of centrifugal cells.

bound or adsorbed on to cellular elements. This was also seen in direct analyses of centrifuged cells, hydrolysed *in toto*, and in determinations of plasma plus cell washings. (For washing, 1 ml of 0.9% NaCl was added to 1 ml of whole blood; this is mixed and centrifuged and the supernatant removed. The cellular precipitate was resuspended in 1 ml 0.9% NaCl, recentrifuged, and the supernatant was again removed. This was repeated three times. Hydroxyproline determinations were performed on the pooled supernatants.)

To ascertain whether this adsorbed hydroxyproline-containing protein represented a form of collagen or C1q, prepared antiserum to human collagen (collagen D, Fig. 1) was used^{8,9}. The antiserum was adsorbed with whole human plasma and then used to visualise collagen in blood smears and clots by immunofluorescence using the indirect method. Smears and sectioned clots were prepared without the use of anticoagulants. The preparations were treated with anticollagen antiserum followed by fluorescein conjugated goat anti-rabbit serum⁶.

In the clots, a prominent diffuse reticulum of fluorescent fibrils interlaced the cellular masses (Fig. 2c,e). The smears showed highly fluorescent platelets and platelets aggregated (Fig. 2a). The clotted extremities of the smear showed a brilliant, if delicate, version of the sectioned clot (Fig. 2b). Here platelet clumps could be distinguished at every fibrillar intersection and could represent the points of initiation for a collagen fibrillar component of clot formation. There was a faint fluorescence surrounding red blood cells which cannot yet be unambiguously attributed to collagen. Collagenase (Worthington) removed the fluorescence reaction from fibrils and platelet clumps (Fig. 2d). Sections were subjected to additional controls including blocking, pretreatment with control rabbit sera, and pre-adsorption of the anti-human collagen serum with the

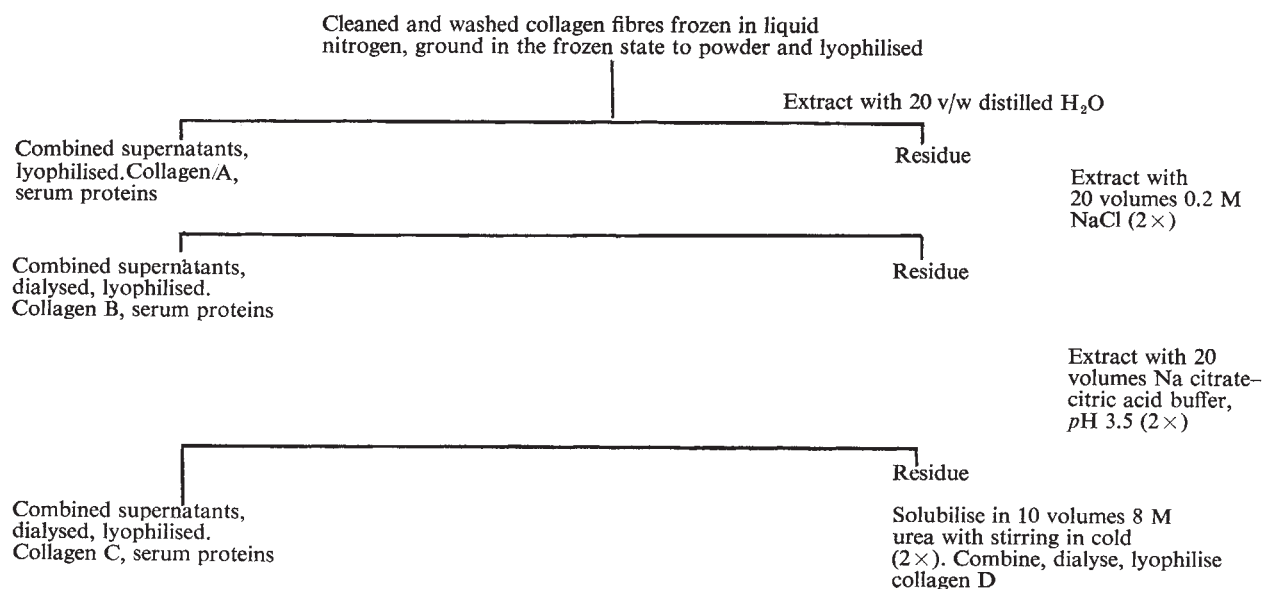
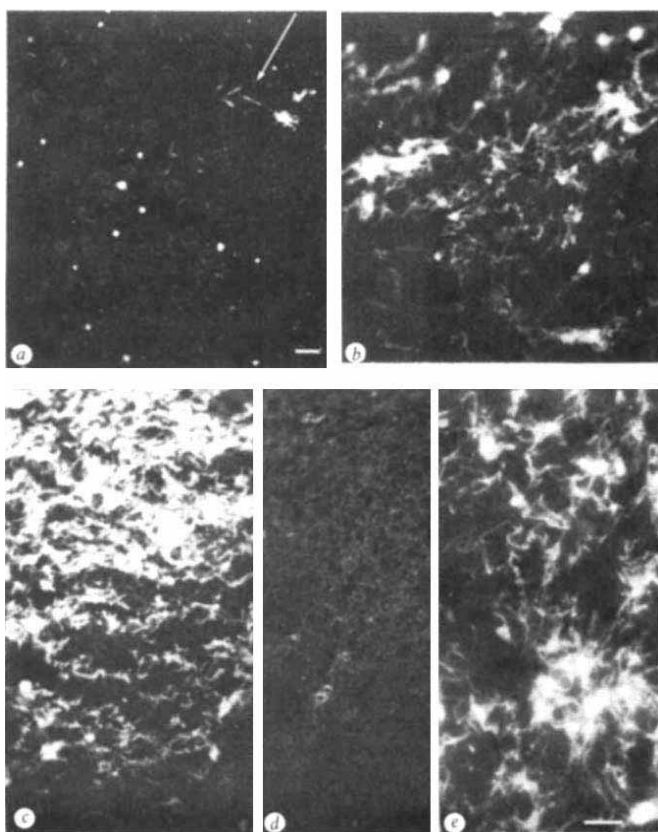


Fig. 1 Fractionation of human achilles tendon.

collagen antigen. Following these steps, the fluorescence reaction for collagen was negative.

The human collagen antiserum was extensively evaluated for specificity. After adsorption with plasma it did not react with serum proteins nor with C1q (M.B.E., unpublished). Amino acid analysis of collagen D was similar, although not identical, to that for human skin collagen¹⁰.

Fig. 2 Immunofluorescence smear and clot preparations of normal human blood stained by the indirect method using a rabbit anti-human collagen serum and a goat anti-rabbit serum conjugated with fluorescein isocyanate. *a*, Blood smear showing platelet fluorescence. At arrow, fine threads of an incipient clot. *b*, Clotted portion of blood smear showing additional collagen component possibly radiating from platelet foci. *c* and *e*, Collagen staining of sectioned clots with maximal appearance of collagen reactive material. *d*, Collagenase control of sectioned clot. Scale bars, 10 μ m.



We had attempted to remove, as far as possible, serum protein contaminants from this preparation. There was evidence for the presence of an unusual post-histidine residue corresponding to the histidinohydroxymerodesmosine described in other insoluble forms of collagen¹¹. It is not known whether the antiserum distinguishes between fibrillar and monomeric collagen.

Isolation procedures to characterise the hydroxyproline-containing protein of blood have been begun by one of us (R.M. and Bezkorovainy, A.). Plasma and supernatants of washed cells were applied to a Sepharose 6B column, using H₂O as eluting medium. Two hydroxyproline-containing peaks were resolved, one appearing in the void volume (molecular weight 10⁶), the other at a molecular weight of 400,000. The eluting medium was changed to 0.1 M NaHCO₃ and two hydroxyproline peaks again appeared, at molecular weights of 400,00 and 300,000. We assume that the 400,000 peak is C1q, while the large protein is some form of collagen which dissociated when the buffer was changed.

The interaction of collagen with platelets *in vitro* has been extensively studied¹²⁻¹⁵, always, however, with the assumption that the *in vivo* source of collagen was subendothelial tissue exposed by vascular injury. The presence of a collagen-like protein already present on the cellular surface must modify this interpretation. Addition of aggregating agents to platelets in platelet-rich plasma showed an almost immediate involvement of amorphous surface material in interplatelet bonds¹⁶. These bonds strongly resemble those seen in Fig. 2*b*. Not only platelets, but leukocytes and erythrocytes interact and aggregate with fibrillar collagen *in vitro*¹⁷. The binding sites involved in this collagen interaction—at least in the platelet and possibly in the leukocyte—also bind C1q and in its presence collagen-induced aggregation is lost. There thus seems to be competition of collagen and C1q for the same binding sites¹⁸⁻²².

In early stages of clotting, a substantial collagen reaction is present on platelets, but a portion remains occult, appearing as insoluble highly reactive fibrils only in areas where clotting had begun and showing maximally in the sectioned clot. Thus an intriguing question is whether this part of the circulating collagen is normally associated with the erythrocyte surface and becomes reactive during clotting. We have not examined smears from anticoagulated blood by immunofluorescence.

The hydroxyproline-containing protein described here

resembles collagen in both antigenic and chemical qualities and is a physiological component of the formed elements of blood. As such, it is certainly involved in their interactions including clot initiation and the possible displacement of Clq from antigen-antibody complexes. This role, and its modifications in situations where collagen becomes abnormally mobilised from tissues, remains to be evaluated in the areas of blood sedimentation, clotting, haemostasis and thrombosis.

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Effect of a single amino acid substitution on stability of conformation of a protein

THE role of individual amino acid residues in stabilising protein molecules is of interest, especially in connection with the mechanism of thermoresistance of thermophilic bacteria¹ and in connection with the physicochemical basis of temperature sensitive mutants used widely in molecular biology studies. This problem has been approached mainly with the use of synthetic polypeptide², chemical modification of natural proteins³, or homologous proteins⁴⁻⁹ from different species. Many mutants of microorganisms are available from genetic studies, however, in which the mutant proteins differ from wild type only at a single amino acid position. Such proteins often differ markedly in stability. We describe here a study of the α -subunit of tryptophan synthetase of *Escherichia coli*. The data suggest that the stability of a protein may be highly correlated with the hydrophobicity of the amino acid residues at some suitable positions. Further studies of this type may clarify the role of individual amino acid residues in stabilising proteins.

Many mutants of *E. coli* producing an α -subunit of tryptophan synthetase with a single amino acid substitution have been isolated¹⁰, and α -subunits of tryptophan synthetase from some *E. coli* mutants have altered stability¹¹. Table 1 shows a comparison of heat inactivation of tryptophan synthetase α -subunits from mutant and wild-type strains. Marked effects were seen when Glu

at position 49 was replaced by Gln (strain *trpA11*) or by Met (*trpA33*); the α -subunit became more labile or more stable, respectively. These results confirm those reported by Maling and Yanofsky¹¹.

α -Subunits were purified from the wild-type strain and the mutant *trpA33*, respectively, using the method of Creighton and Yanofsky¹² with a slight modification. Purified samples gave a single band on polyacrylamide gel electrophoresis. Circular dichroism (CD) spectra (200-250 nm) of the purified α -subunits from the wild type and *trpA33* were mutually not distinguishable and coincided with the results of Heyn and Weisheit¹³ on the wild-type protein. The heat inactivation of the purified samples coincided with those of crude extracts. We can therefore assume that the difference in thermostability of crude enzymes in Table 1 reflects the difference in that of the α -subunit molecule itself, which

Table 1 Heat inactivation of α -subunit of tryptophan synthetase of *E. coli*

<i>trp</i> Strain	Position of amino acid substitution ¹⁰	Remaining activity (%)
A218	22 Phe → Leu	10 ± 1
A11	49 Glu → Gln	22 ± 3
A33	49 Glu → Met	88 ± 13
A446	175 Tyr → Cys	60 ± 1
A487	177 Leu → Arg	16 ± 7
A223	183 Thr → Ile	35 ± 2
A23	211 Gly → Arg	13 ± 2
A46	211 Gly → Glu	76 ± 13
A187	213 Gly → Val	12 ± 4
A78	234 Gly → Cys	40 ± 8
A58	234 Gly → Asp	85 ± 10
A169	235 Ser → Leu	30 ± 4
B8	(Wild type)	49 ± 4

Cells suspended in pH 8.0, 0.1 M Tris-HCl buffer were disrupted in a Branson Sonifier B-12 sonic oscillator. The supernatant solutions obtained by centrifugation for 2.5 h at 80,000g were used for the heat inactivation study. These mutant proteins are inactive in the conversion of indole-3-glycerol phosphate to tryptophan, but retain the activity for the conversion of indole to tryptophan in the presence of pyridoxal phosphate, serine, and β -subunit of tryptophan synthetase of *E. coli*¹⁴. This latter activity was measured by the disappearance of indole after reaction at 37 °C for 20 min¹⁴. The β -subunit was extracted from mutant strain *trpA2* of *E. coli*. Heat treatment was done for 20 min at 58 °C.

is due to replacement of a single amino acid residue. The conformational changes of the α -subunits on heating were examined by measuring the CD values at 222 nm, where the CD spectra of the α -subunits showed a large negative Cotton effect. After incubation for 60 min at 60 °C in pH 8.0, 0.1 M Tris-HCl buffer, the per cent of remaining ellipticity at 222 nm of α -subunits from the wild type and *trpA33* was 66.5 and 82.0% respectively. After cooling for 10 min at 25 °C, the ellipticity of α -subunits from the wild type and *trpA33* recovered to 81.5 and 94.0%, respectively. When 0.2 mM dithioerythritol was present in the sample solutions, changes of ellipticity on denaturation were similar to those seen in its absence, but the recovery of the ellipticity was stimulated remarkably in both wild-type and mutant proteins. These results show that the conformation of the α -subunit from *trpA33* is more stable to heat than that of the wild type. It must be pointed out that the activity of α -subunit may recover to some extent during assay (20 min at 37 °C) after heat treatment. Data on heat inactivation of enzymatic activity may therefore only partially reflect conformational stability of the α -subunit.

We examined urea denaturation of the two proteins as an indication of stability, and also as the simplest procedure¹⁵ for estimating the free energy change of unfolding. Figure 1 shows a comparison of urea denaturation of the purified α -subunits of the wild type and the mutant *trpA33* measured by the change in CD at 222 nm. The mutant *trpA33* protein was more resistant to urea than that of the wild type, corresponding to the relative stabilities on heat denaturation. From far-ultraviolet CD spectra, the two proteins could be considered to be completely denatured in 8 M urea. The denatured proteins were renatured by diluting (Fig. 1),

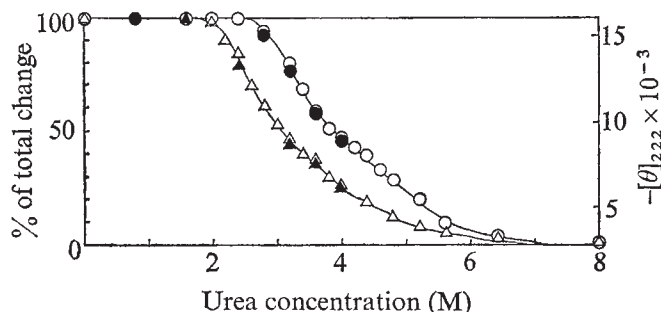


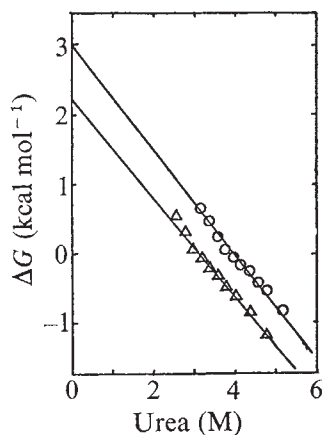
Fig. 1 Urea denaturation of the α -subunits of the wild type (Δ , $\blacktriangle$) and the mutant *trpA33* ($\circ$, $\bullet$). Temperature 25 °C, pH 8.0, 0.025 M Tris-HCl buffer. The solution contained 0.1 mM EDTA and 0.2 mM dithioerythritol. Open symbols represent the ellipticity at 222 nm after incubation for 60 min in urea solutions at various concentrations. Filled symbols represent the measurement of the samples diluted after exposure to 8 M urea solution to demonstrate the reversibility of the denaturation. CD measurements were carried out with a Jasco J-20 recording spectropolarimeter.

and enzymatic activity recovered completely. Urea denaturation is thus reversible in these conditions. The midpoints of urea denaturation curves at 25 °C were 3.1 and 3.9 M urea for the wild type and the mutant *trpA33*, respectively.

We analysed the data shown in Fig. 1 assuming that the urea denaturation can be approximately represented by an equilibrium between a unique native form and a completely unfolded form^{8,9,15}. Figure 2 shows the dependence of the free energy change of unfolding on the urea concentration. By extrapolating to zero concentration, the free energy changes of unfolding in water for the α -subunit of the wild type and the mutant *trpA33* are calculated to be 2,290 and 3,040 cal mol⁻¹, respectively. The difference between the wild type and the mutant *trpA33*, 750 cal mol⁻¹, amounts to about 30% of the free energy change of unfolding for the wild type.

X-ray diffraction studies have shown that the replacement of Glu 49 by Met caused little alteration of the conformation of the α -subunit¹⁶. Our results, taken together with the above data, show that a single amino acid substitution increased the stability of the molecule without a gross change in conformation. The side chain contribution of an amino acid to free energy change for transfer from ethanol to water has been estimated to be 1,300 and 550 cal mol⁻¹ for Met and Glu, respectively¹⁷. The difference between them, 750 cal mol⁻¹, coincides with the difference of the free energy change of unfolding of the wild type and the mutant *trpA33* proteins obtained here. This suggests that the increase in stability

Fig. 2 Effect of urea concentration on the free energy change of unfolding for α -subunits of the wild type (Δ) and the mutant *trpA33* ($\circ$). ΔG for two-state transition is given by the following equation: $\Delta G = -RT \ln K$. An equilibrium constant, K = (concentration of unfolded molecule)/(concentration of native one), is calculated from the data shown in Fig. 1, using only those points where K is between 0.2 and 5.0 (ref. 15). The lines in the figures were calculated by the method of least squares.



of the mutant *trpA33* protein compared with the wild type might be accounted for by the difference of hydrophobicity of Glu and Met, if we assume that the position 49 is somehow buried in the hydrophobic interior of the molecule. Although hydrophilic residues such as glutamic acid are usually located on the surface of protein molecules, there are exceptions, especially when the residues are in the active sites of enzymes¹⁸. In the case of Glu 49 of the α -subunit of tryptophan synthetase, its substitution by Met results in loss of activity with indole glycerolphosphate and serine as substrate, though not with indole and serine¹⁴. We may therefore assume that it is in the active site of the enzyme and hence in a largely hydrophobic interior environment, which may be simulated by ethanol or similar solvents, and in which partners for hydrogen bonding are at hand.

Our results suggest that the stability of an enzyme may be greatly increased by the increase in hydrophobicity by substitution of a few suitable amino acid residues. Stability of enzymes from thermophiles could be explained by similar mechanism.

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Preponderance of synonymous changes as evidence for the neutral theory of molecular evolution

ACCORDING to the neutral mutation-random drift hypothesis of molecular evolution and polymorphism^{1,2}, most mutant substitutions detected through comparative studies of homologous proteins (and the nucleotide sequences) are the results of random fixation of selectively neutral or nearly neutral mutations. This is in sharp contrast to the orthodox neo-Darwinian view that practically all mutant substitutions occurring within species in the course of evolution are caused by positive Darwinian selection³⁻⁵. This paper shows that by comparative studies of messenger RNA (mRNA) sequences reliable estimates can be obtained of the evolutionary rates (in terms of mutant substitutions) at the third positions of the codon, and that the estimates conform remarkably well with the framework of the neutral theory.

Salser *et al.*⁶ have presented a comparison of homologous parts from the fragments of the human and rabbit haemoglobin mRNA sequences. Among 53 nucleotide positions that can be compared, there are six base differences, of which only one leads to amino acid difference. Their Table 5 shows that among 17 third nucleotide positions of the codon which can be compared

between the two species, there are 5 nucleotide differences. To estimate the number of nucleotide substitutions per site (K) that have occurred in the course of evolution since the divergence of the rabbit and human lineages, I use the following formula which converts the observed difference into the estimate of evolutionary divergence

$$K = -\frac{3}{4} \log_e(1 - 4/3 \lambda) \quad (1)$$

In this formula, λ is the observed fraction of the sites by which two homologous sequences differ from each other (for details, see ref. 7); K includes superimposed and reverted mutant substitutions. The rate of nucleotide substitution per site per year can then be obtained by $k_{\text{nuc}} = K/(2T)$, where T is the time (yr) since the divergence of the two lineages. Also, the standard error (σ_K) of K may be computed using a formula given in ref. 7. Letting $\lambda = 5/17$ in equation (1), we obtain $K = 0.373$. The corresponding standard error (σ_K) is 0.182. Thus, taking $T = 8 \times 10^7$, we obtain $k_{\text{nuc}} = (2.3 \pm 1.1) \times 10^{-9}$ (the present estimate should be statistically more reliable than the corresponding estimate given in ref. 6). This is a very high evolutionary rate, comparable with that of the fibrinopeptides. If we denote by k_{aa} the rate of amino acid substitutions per site, it is known that for fibrinopeptides $k_{\text{aa}} = (4 \sim 9) \times 10^{-9}$ (see refs 8, 9). This figure, when converted into the nucleotide substitution rate, is $k_{\text{nuc}} = (1.8 \sim 4.0) \times 10^{-9}$. In addition, a direct comparison of human and rabbit fibrinopeptide A (using data given in ref. 8) gives $k_{\text{aa}} = 5.2 \times 10^{-9}$ leading to $k_{\text{nuc}} = 2.3 \times 10^{-9}$.

A similar but more interesting report comes from Grunstein *et al.*¹⁰ who compared histone H4 (that is, histone IV) mRNA sequences of two sea urchin species *S. purpuratus* and *L. pictus*. It is well known that histone H4 is by far the most highly conserved protein (with $k_{\text{aa}} = 0.006 \times 10^{-9}$ (ref. 8)). Their report¹⁰ now shows, however, that many synonymous changes (that is, the changes that do not lead to amino acid changes) have occurred in the gene coding for this protein in the course of evolution. From their Table 3, showing 84 nucleotide sites that can be compared between the messenger sequences of the two species, there are ten base differences, of which nine are located at the third positions of the codon. Actually, there are 27 third nucleotide positions which can be compared between these two species and there are nine nucleotide differences. Thus, we obtain $K = 0.440$ and $\sigma_K = 0.163$. According to Grunstein *et al.*, these sea urchins shared a common ancestor approximately 6×10^7 yr ago. Therefore, letting $T = 6 \times 10^7$, we obtain $k_{\text{nuc}} = (3.7 \pm 1.4) \times 10^{-9}$ as an estimate of the average evolutionary rate at the third nucleotide position of the codon in the histone H4 gene. It is remarkable that, in this gene, synonymous mutant substitutions have occurred with the highest known rate, in spite of the fact that the amino acid substitutions have occurred in the corresponding protein with the lowest known rate.

How do these observations bear relation to the neutral theory? Let k be the rate by which mutant genes are substituted in the species in evolution. If a certain fraction f_0 of the molecular mutants are selectively neutral and the rest are definitely deleterious (assuming that definitely advantageous mutations are negligible in frequency), then for neutral alleles, the rate of mutant substitutions is equal to the mutation rate¹. Therefore

$$k = v_T f_0 \quad (2)$$

where v_T is the total mutation rate. Now, among the prominent features¹¹ of molecular evolution the following two are particularly noteworthy: (1) for a given protein, the rate of evolution is roughly constant per year and (2) the molecules or parts of molecules that are subject to less functional constraints evolve (in terms of mutant substitutions) faster.

As to the first feature, the neutralists assume that for a given molecule f_0 is roughly constant. Recently, some authors, such as Fitch and Langley¹², have emphasised the non-constancy, but

their results show, in agreement with our earlier estimate¹³, that the variance of evolutionary rates among lines is only about 2.6 times as large as that expected from chance fluctuations. It is possible that there is delicate fluctuation of intrinsic evolutionary rate around the mean, as caused by shifting of the molecular constraint as amino acids are substituted one after another in various parts of the molecule. This means that the probability f_0 of a mutation being neutral is not strictly constant but fluctuates around its characteristic mean. Also, as pointed out by Ohta¹⁴, if nearly neutral but very slightly deleterious mutations are prevalent, the evolutionary rate fluctuates as the population goes through a series of bottlenecks. As to the difference of evolutionary rates among molecules or parts of molecules, the neutralists assume that the probability of a mutational change being neutral depends on functional constraints. Namely, the weaker the functional constraint, the larger the probability (f_0) of a random change being selectively neutral, with the result that k in equation (2) gets larger. According to this explanation, the maximum evolutionary rate is attained when $f_0 = 1$, that is, when all the mutations are neutral. Now, the high evolutionary rates observed at the third position of the codon can be explained from the neutral theory by assuming that the majority of synonymous changes are selectively neutral. Note that roughly 2/3 of random nucleotide substitutions at the third position of the codon are synonymous. The possibility of synonymous nucleotide changes being selectively neutral was discussed extensively in an early paper² on the neutral theory. Of course, it is possible that not all synonymous mutations are completely neutral, but the possibility is very high that, on average, synonymous changes are subject to natural selection very much less than the mis-sense mutations. On the other hand, if we adhere to the selectionist position that practically all the mutant substitutions in evolution are caused by positive natural selection, there can be no upper limit to the evolutionary rate at the molecular level (as directly set by the mutation rate v_T), and there is no reason to believe that the rate of evolution is uniform even approximately for a given molecule among different species.

In my opinion, various observations suggest that as the functional constraint diminishes the rate of evolution converges to that of the synonymous substitutions. If this is valid, such a convergence (or plateauing) of molecular evolutionary rates will turn out to be strong supporting evidence for the neutral theory.

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Inheritance of recombinant HLA-GLO haplotype suggesting the gene sequence

THE HLA region in man has been assigned to the short arm of chromosome 6 (ref. 1). Close genetic linkage has been shown between *HLA* and genes involved in the synthesis of the second^{2–3} and fourth⁴ components of complement as well as structural variation of the glycine-rich B-glycoprotein (GBG or Bf)⁵. Two enzymes, phosphoglucomutase-3 (PGM₃)^{6–7} and glyoxalase I (GLO)^{1,8–12}, and two blood group antigens, Chido¹³ and Rodgers¹, have been shown to be in this linkage group. Here we describe a

Table 1 M. family MLC testing stimulating cells

Responding cells HLA genotypes			A ×	B ×	C ×	D ×	E ×	F ×	Unrelated pool†	Reference stimulation‡
A	Father (TM)	A29-B12 c.p.m.*	8,225	99,415	95,191	91,244	96,729	90,928	90,046	81,818
		RR	0	87	106	101	108	101	100	
		A2 -B12 SIR	1	9.7	11.6	11.1	11.8	11.1	10.9	
B	Mother (MM)	A2 -B21 c.p.m.	51,554	3,792	49,346	61,240	48,821	48,739	83,394	79,603
		RR	60	0	57	72	57	57	100	
		A32-B7 SIR	13.6	1	13.0	16.0	12.9	12.9	22.0	
C	Sibling I	A29-B12 c.p.m.	61,135	20,893	992	64,033	66,456	44,478	73,454	72,462
		RR	83	28	0	87	90	60	100	
		A32-B21 SIR	61.6	21.1	1	64.5	67.0	44.8	74.0	
D	Sibling II (CM)	A2 -B12 c.p.m.	42,000	17,952	37,808	2,508	7,499	10,934	65,151	48,526
		RR	63	25	56	0	0	13	100	
		A32-B7 SIR	16.3	7.0	14.7	1	2.9	4.2	25.3	
E	Sibling III (MM)	A2 -B12 c.p.m.	45,654	11,956	16,886	7,784	1,991	1,485	51,518	63,007
		RR	91	19	49	10	0	0	100	
		A2 -B21 SIR	15.3	4.0	9.0	2.6	1	0.5	17.2	
F	Sibling IV (MaM)	A2 -B12 c.p.m.	57,514	19,058	38,047	9,053	4,999	2,068	65,075	63,007
		RR	88	27	57	11	5	0	100	
		A2 -B21 SIR	27.8	9.2	18.4	4.4	2.4	1	31.5	

RR, relative response = $\frac{\text{test values (c.p.m.)—autologous stimulation (c.p.m.)}}{\text{response to unrelated pool—auto. stim (c.p.m.)}}$

SIR, stimulation index ratio = $\frac{\text{test value (c.p.m.)}}{\text{autologous stimulation (c.p.m.)}}$

*Tritiated thymidine incorporation.

†Pool of three unrelated controls.

‡Pool—autologous control.

family with an intra-HLA recombinant sibling and an HLA-GLO recombinant sibling. The mother is heterozygous at the HLA-A and -B loci, homozygous for HLA-DW2, and heterozygous at the GLO locus, thus permitting identification of an HLA-GLO recombinant in one of her children. Our investigations suggest that the locus for GLO polymorphism is on the centromeric side of HLA-B on the short arm of chromosome 6.

Table 1 illustrates the family mixed lymphocyte culture results¹⁴ showing that the lymphocytes of the mother only weakly stimulated those of her children, but in the reciprocal MLC reactions, her lymphocytes responded briskly to their. The sero-identical siblings III and IV are non-responsive in mutual MLC reactions, indicating identity at HLA-D. Interestingly, sibling II shows low mutual MLC responses with the HLA-D identical siblings III and IV.

These observations were corroborated by the HLA-D typing (Table 2). The family members were tested against homozygous typing cells representing the HLA-DW1-4 clusters as defined by the Sixth International Histocompatibility Workshop 1975; two HTC (FS and KL) which do not fall within the presently defined DW clusters, and one HTC (JB-12) which corresponds to the specificity

LD 107. The HLA-D typing results were analysed by the relative response and cluster methods⁴. The mother types as a HLA-DW2 and seems to be homozygous. The siblings II, III and IV are HLA-D identical (DW1-DW2) and sibling I inherited an HLA-A to -B recombinant (HLA-DW2-LD-107).

Slightly higher MLC reactivity than one would expect with complete HLA-D identity is observed when sibling II serves as either stimulator of or responder to the sero-identical siblings III and IV. Although all three siblings type as HLA-DW1, DW2, the maternal haplotypes apparently carry slightly dissimilar DW2 determinants. This interpretation is consistent with evidence previously presented that the HLA-D region is not governed by a single gene, but more probably by a group of closely linked genes. Thus, the respective maternal D determinants may be overlapping or one may represent an inclusion of the other determinant⁴. Alternatively, the two maternal haplotypes may carry the same major HLA-D determinants, but differ at minor determinants at another locus¹⁵ or may differ at response genes¹⁶. Furthermore, back stimulation may be present to minor HLA-D determinants¹⁷.

The Bf typing¹⁸ gives rise to three common phenotypes

Table 2 Chromosome 6—gene markers of M family

Subject	Relationship	Haplotype designation	HLA-A*	HLA-B*	Bf	HLA-D	GLO	CHIDO
TM	Father	a	A2	B12	S	DW1	2	Ch (a+)
MM	Mother	b	AW29	B12	F	JB-12	2	Ch (a+)
		c	AW32	B7	S	DW2	2	
BM	SibI	d	A2	BW21	S	DW2	1	Ch (a+)
		b	AW29	B12	F	JB-12	2	
		c-d (HLA-A-B recomb)	AW32	BW21	S	(LD-107) DW2	1	
CM	SibII	a	A2	B12	S	DW1	2	Ch (a+)
MiM	SibIII	c	AW32	B7	S	DW2	2	Ch (a+)
		a	A2	B12	S	DW1	2	
		d,c (HLA-D/GLO recomb)	A2	BW21	S	DW2	2	
MaM	SibIV	a	A2	B12	S	DW1	2	Ch (a+)
		b	A2	BW21	S	DW2	1	

*HLA determined by lymphocytotoxicity (see ref. 21).

(SS, FS and FF) determined by the relative mobility of the slow or fast migration bands to the anode of the electrophoretic plate. The father and sibling I demonstrates the FS phenotype; the mother and siblings II, III and IV the SS phenotypes. Thus, the F component is linked to the HLA-AW29, B12 haplotype of the father. Since the mother is homozygous for the S determinant, the Bf typing is not informative in following the crossovers involving the maternal chromosomes in the recombinant siblings I and II.

Glyoxalase-1 (EC 4.4.1.5) catalyses the formation of S-lactoyl glutathione from glutathione and methylglyoxal. Genetic polymorphism of this enzyme in red blood cell lysates was first reported by Kompf, *et al.*¹⁰. They described three different starch gel patterns representing homozygosity and heterozygosity for two alleles, GLO¹ and GLO², with frequencies of about 0.40 and 0.60, respectively. The method of Kompf, *et al.*¹⁹ was used for this study, except a phosphate buffer was used²⁰. Since the enzyme is a dimer, the heterozygote pattern has three major zones (two homodimers and one heterodimer), while the homozygote patterns contain only one major band each. Thus, the phenotypes are unequivocal, even though they are difficult to photograph, because the sites of catalytic activity appear as a lighter shade on the surrounding darker blue background. Figure 1 shows the three common phenotypes.

Several investigators^{1,8-10} have reported genetic linkage of GLO and HLA on chromosome 6. Due to the inability to establish linkage between PGM₃ and GLO, Kompf¹¹ suggested that GLO might be located on the side of the HLA-D locus at a distance of about 15 map units. The

sex-dependent difference in recombination frequency noted by Lamm *et al.* for PGM₃ was not observed by Weitkamp *et al.*⁹ for GLO, an observation consistent with the postulation by Kompf¹¹ that GLO may be located in the HLA-A side of the chromosome. Subsequent family studies, however, show GLO segregates with HLA-B^{1,9-10}. Weitkamp *et al.*¹ in studies of linkage between HLA, Bf and GLO suggested the GLO is closer to Bf than to HLA. Meera Khan *et al.*¹ in other linkage studies suggested that GLO is situated between PGM₃ and HLA. Furthermore, from the estimated recombination frequency between HLA and GLO of 0.15 (ref. 8), Pretorius *et al.*¹⁰ postulated the GLO locus to be located outside of HLA-D.

Assuming the least number of recombinants in the family described herein, the GLO locus seems to be outside the locus for HLA-B. The placement of the GLO locus on the centromeric side of HLA-B is strengthened by the observation of MLC non-reactivity between siblings III and IV indicating their HLA-D identity. Thus, the maternal haplotypes are apparently HLA-AW32-B7-DW2-GLO2 and HLA-A2-BW21-DW2-GLO1. Sibling III inherited a recombinant haplotype HLA-A2-BW21-DW2-GLO2 with the recombination occurring somewhere between the HLA-B and GLO. Our investigation therefore suggests that the most probable order of loci on the short arm of chromosome 6 proceeding toward the centromere is: HLA-A, HLA-B, HLA-D, GLO.

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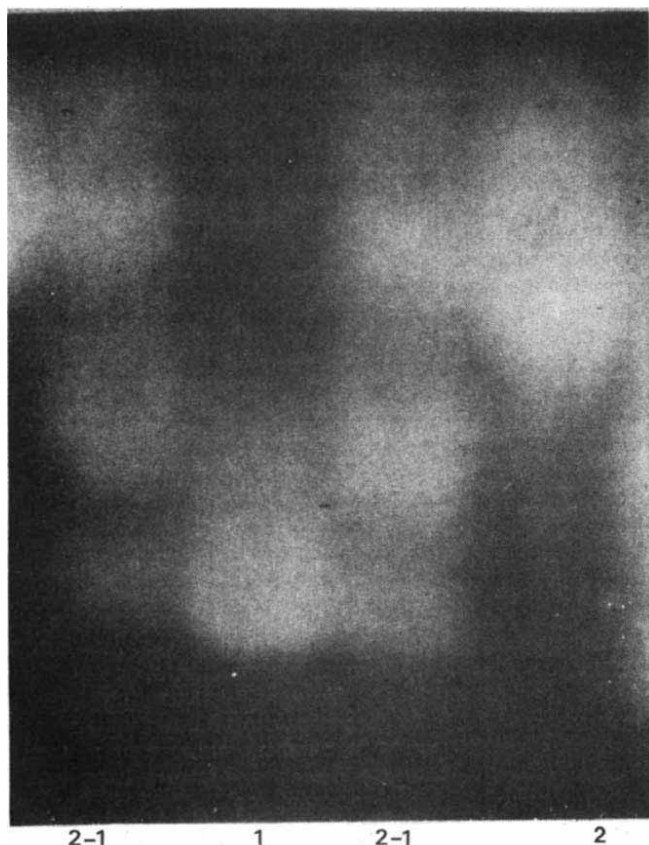
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Fig. 1 Starch gel electrophoretic patterns of the three common glyoxalase-1 phenotypes, GLO 1, 2-1, and 2. The sites of enzyme activity are lighter coloured than the darker staining background.



Structure-function relationship of 5' non-coding sequence of rabbit α - and β -globin mRNA

RETICULOCYTES make essentially equimolar amounts of α - and β -globin as required for the correct assembly of haemoglobin. It has been shown, however, that α and β mRNAs differ in their rate constant for ribosome attachment^{1,2}. In particular α -globin mRNA initiates protein synthesis only 60% as frequently as β -globin mRNA (refs 3–5). The 5' non-coding region is thought to be involved in the binding of the mRNA to the ribosome for the correct initiation of protein synthesis. A comparative study of the sequences of the 5' non-coding regions of α - and β -globin mRNA may show whether their differential rate of initiation can be ascribed to specific features in the mRNA structure or whether it is a function of some other part of the translational machinery. Recently the complete sequence of the 5' non-coding region of rabbit β -globin mRNA has been elucidated⁶. I report here the complete sequence of the 5' non-coding region of rabbit α -globin mRNA, determined following essentially the same methodology.

A ³²P-labelled complementary DNA (cDNA) of the 5' non-coding region of both α - and β -globin mRNA was prepared using the synthetic deoxyhexanucleotide primer d(G-C-A-C-C-A) complementary to the codons for the Met-Val sequence common to both proteins and avian myeloblastosis virus (AMV) reverse transcriptase.

The cDNA was fractionated by one-dimensional homochromatography (see Fig. 1a). Bands 2, 3 and 5 were identified as cDNA of the 5' non-coding region of β -globin mRNA by their characteristic depurination fingerprints⁶. Bands 1 and 4 were tentatively classified as cDNA of the 5' non-coding region of α -globin mRNA because their intensity relative to the β bands increased when a template enriched in the α component

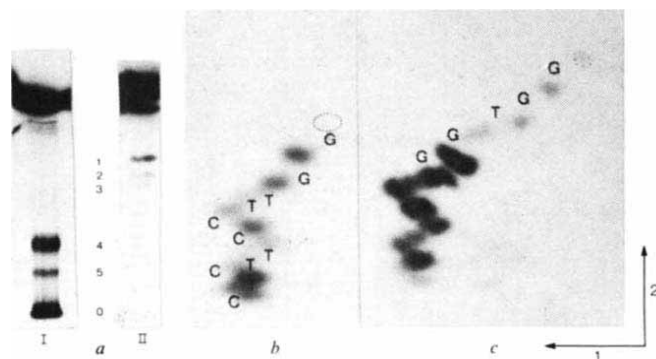


Fig. 1 a, Autoradiograph of a one-dimensional homochromatography fractionation of cDNA (ref. 7). Radioactive cDNA was prepared *in vitro* with AMV reverse transcriptase using each α -³²P-labelled deoxynucleoside triphosphate (dNTP) of high specific activity in turn and the other three unlabelled. The synthesis was carried out with all four dNTPs present (I), or in the absence of dATP (II). O denotes the origin, and the strong band at the top is unreacted dNTP. The reaction conditions were identical to those previously described⁶, except that the primer used was the hexanucleotide d(G-C-A-C-C-A) and the template was a mixture of α - and β -globin mRNA (a gift from Dr N. J. Proudfoot) obtained from the post-ribosomal supernatant that had been shown to be enriched in the α component⁸. AMV reverse transcriptase was a gift of Dr J. W. Beard. Bands 1 and 4 are cDNA of α -globin mRNA 5' non-coding region; band 1 ends before the first dA residue and band 4 is the full length copy. Bands 2 and 5 are cDNA of β -globin mRNA 5' non-coding region; band 2 ends before the first dA residue and band 5 is the full length copy. b, c, Two-dimensional fingerprints of a partial venom phosphodiesterase digest of band 1 labelled with dGTP (b) or dTTP (c). The sequence d(5') [T]G-G-T-G-G-T-T-C-C-T-T-C-T-C(3') was deduced from the relative mobilities of the intermediates. The absence of radioactive products after the last G shift in (c) is interpreted as the next residue being the dT adjacent to the primer.

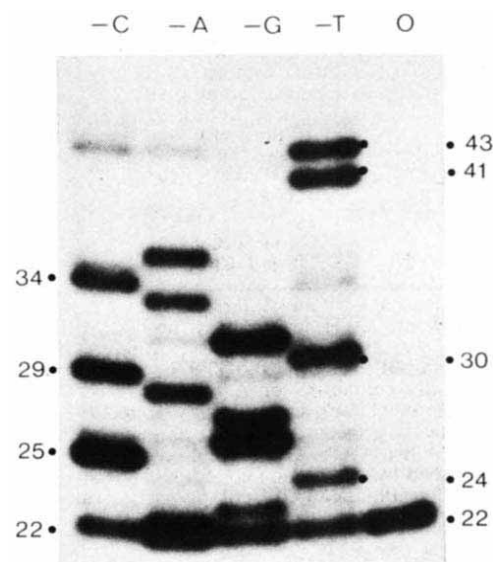
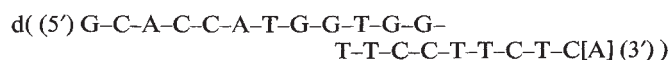


Fig. 2 Gel analysis of partially minus reactions. Band 1 was eluted from the homochromatography, desalted through a Sephadex G-100 column and freeze dried. The cDNA was then dissolved and re-annealed with mRNA. This preparation was divided into four aliquots, and 50 nM 'minus' reactions⁹ were performed. Each 'minus' mixture contained three dNTPs at 50 μ M concentration and one dNTP in turn (the limited one, so called 'minus') at 50 nM concentration. In this way it was possible to obtain from a single product (band 1) specific 'pile-ups' of radioactivity in positions corresponding to the limiting dNTP. The reaction mixtures were fractionated in 7 M urea 12% polyacrylamide gel. The slots marked -C, -A, -G, -T denote that the reaction analysed was carried out with the corresponding dNTP at a concentration of 50 nM. Slot O is a control lacking the enzyme. The bands correspond to positions in the sequence where the limiting dNTP is present. The numbering refers to the cDNA sequence shown in Fig. 3. Longer exposed films clearly showed bands at position 38–39 in the -A slot and at position 32 and 42 in the -G slot.

was used. Definitive evidence for this assignment is discussed below.

Nearest neighbour analysis carried out for band 1 showed that only when the label was α -³²P-dTTP was radioactive dAp found; dCTP and dGTP failed to label dAp (see Table 1). As band 1 was synthesised in the absence of dATP, and therefore lacked any internal dA residues, it was concluded that the last residue of the primer was the one labelled, as expected if the initiation codon was A-U-G. Depurination¹⁰ of bands 1 and 4 provided the data summarised in Table 1. Partial digestion with venom phosphodiesterase of the cDNA of band 1 was carried out; the two-dimensional fingerprint is shown in Fig. 1b and c. The cDNA sequence d(5') G-G-T-G-G-T-T-C-C-T-T-C-T-C(3') can be read off from the characteristic mobility shifts⁹. Note the sharp difference in spot intensity after the first G shift. This unusual pattern may be an indication of some structural feature (hairpin loop?) in this short cDNA oligonucleotide.

Combining data from depurination, nearest neighbour analysis and partial venom phosphodiesterase digestion, the sequence of band 1 cDNA was established as



To obtain the sequences between the end of band 1 and the end of band 4 (possibly the full length cDNA) a new gel sequencing technique was used¹⁹. This is an adaptation for RNA sequencing of the 'plus' and 'minus' system developed for Φ X DNA sequencing¹¹. It consists of the extension of a mixture, as heterogeneous as possible, of different transcript lengths in the complete absence of each one of the four dNTPs in turn. In this case a slight variation was introduced to overcome the problem

Table 1 Depurination and nearest neighbour analysis data from bands 1 and 4 cDNA (Fig. 1a)

Band no.	Input label	Base composition of depurination products (deduced from their position)	Nearest neighbour analysis data			
			C	A	G	T
1	dGTP	T[G]	0	0	1	1
1	dTTP	T ₅ C ₄ *, T	2	1	2	2
1	dCTP	T ₅ C ₄ *	1	0	0	3
4	dATP	C ₂ [A]; T ₅ C ₄ [A]*	—	—	—	—
4	dGTP	T[G]; T C[G]	—	—	—	—

Depurination data—5' and 3' terminal phosphates of depurination products are omitted. [] denotes that the base within brackets was deduced by nearest neighbour considerations. Thus depurination products obtained from α -³²P-dGTP or α -³²P-dATP input label oligonucleotides contain G or A residues respectively on their 3' sides. Nearest neighbour analysis—this section is divided into four columns indicating the products of analysis Cp, Ap, Gp and Tp. These are denoted by C A G T respectively. Ratios of product yields were established by scintillation counting of the spots.

*Base composition deduced from the partial venom digests shown in Fig. 1b.

of nonspecific priming. The extension of a single product (the α -globin mRNA specific product—band 1) was carried out in partially minus conditions (each of the four dNTPs, in turn, at limiting concentrations—see Fig. 2). The band pattern produced allowed the reading of the cDNA sequence as d(5') A-G-T-C-G-G-A-C-T-G-G-A-C-C-A-X-A-A-X-T-G-T (3') where X represents unknown residues, their presence deduced by the spacing of the bands. The last ten residues overlap with the sequences already reported for the 5' end of α -globin mRNA¹², thus confirming the identity of the priming site. It is a ten-base overlap with two ambiguities. Because of the clear overlap it was not necessary to establish the nature of the

X residues in this study as they had been defined by others. The complete cDNA sequence deduced is shown in Fig. 3a.

The 5' non-coding regions of α - and β -globin mRNA (Fig. 3b) are substantially dissimilar in size and nucleotide composition. These sequences have only a few common features: residues 1–6 are identical¹³ and there is a purine-rich region centred six to eight residues to the left of the A–U–G initiating codon.

It has been suggested⁶ (although critical evidence is still lacking) that the region around the A–U–G initiating codon of β -globin mRNA and brome mosaic virus (BMV) RNA interact with the 3' terminal sequence of 18S rRNA. This is similar but not identical to the interaction between the 5' non-coding region of mRNA and 16S rRNA in bacteria^{14,15}. For α -globin mRNA the same interaction is possible at positions 37–41 (Fig. 3), involving the first two triplets of the coding region (common to both messengers) but forming one base pair less than β -globin mRNA. Another possible region of interaction with 18S rRNA is at nucleotides 8–12 (Fig. 3), but this is less attractive for two reasons. First, in the known bacterial examples¹⁶ the interaction of the mRNA with 16S rRNA occurs at a maximum distance of 10 bases from the initiation codon, while here if an interaction were to occur it would be 24 bases away. Second, it has been shown that the A–U–G is in a rather central position of the ribosome-protected fragment in the case of β -globin mRNA^{16,17}. It should be noted that residues 8–12 are in the stem of the proposed hairpin loop (see below).

An obvious problem with the proposed mRNA–18S rRNA interaction around the initiation codon is that both the Met-tRNA_i and the 18S rRNA would compete for the A–U–G sequence of the mRNA. This would be unlike the situation in *Escherichia coli* where the interacting site of the mRNA is displaced approximately ten nucleotides to the left of the initiation codon. The difficulty may be resolved if the two interactions occur sequentially rather than concurrently. Other parts of the extensive 5' non-coding region may also be involved

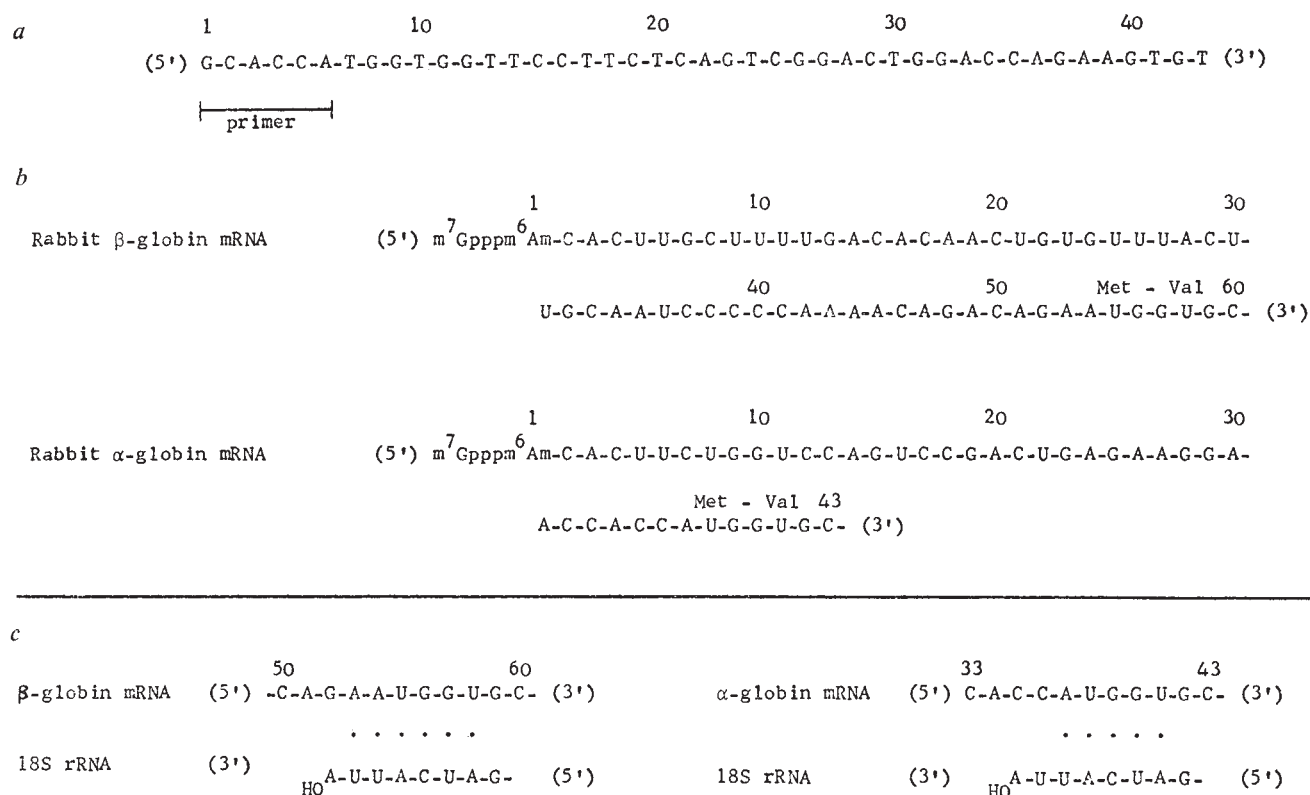


Fig. 3 *a*, Sequence of the cDNA. Numbers indicate the distance of the nucleotide at that position from the 5' end of the primer. The sequence between positions 34 and 43 has been previously reported¹². *b*, Sequence of the 5' non-coding region of rabbit α - and β -globin mRNA. Numbers indicate the distance of the nucleotide at that position from the 5' m⁷Gppp structure. *c*, Base pairing of α - and β -globin mRNA with the known 3' end sequence¹³ of rabbit 18S rRNA.

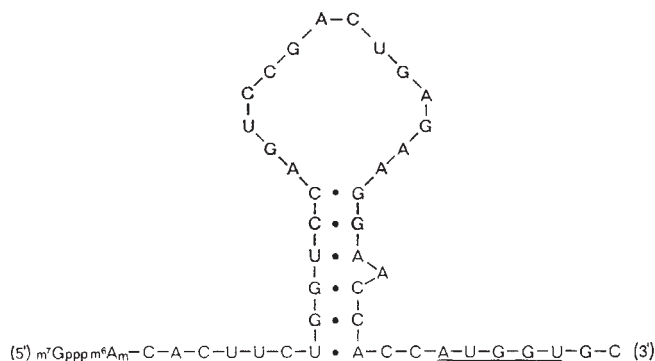


Fig. 4 Possible conformation of the 5' non-coding region of α -globin mRNA. The estimated stability of the hairpin loop is discussed in the text. The underlined sequence is complementary to the 3' end of rabbit 18S rRNA (see Fig. 3).

in other RNA-RNA interactions; for example, by base pairing with internal positions of 18S rRNA.

A number of possible secondary structure models can be drawn by maximising the extent of base pairing. In particular for α -globin mRNA a hairpin loop can be constructed (Fig. 4) that has a large negative free energy (ΔG (25 °C) = -7.6 kcal), estimated according to the rules proposed by Tinoco *et al.*¹⁸. Other secondary structures can be drawn that partially 'mask' the postulated 18S rRNA interacting region (underlined in Fig. 4), but are less stable than the proposed loop. For β -globin mRNA there is no indication of stable secondary structure⁶. The best hairpin loop that can be drawn (not shown) has a negative free energy six times smaller than the α -globin mRNA conformation shown in Fig. 4 and is considered to be unstable, or only marginally stable. It is interesting to note that the α -globin mRNA hairpin loop (Fig. 4) involves about two-thirds of the 5' non-coding region, and most of the adjacent sequence is common with that of β -globin mRNA (the first six nucleotides and the first two codons, see Fig. 3).

The purpose of this study was to elucidate the sequence of the 5' non-coding region of α -globin mRNA. It was hoped that a comparison of this sequence with its β counterpart would provide a clue to understanding the molecular basis of their differential rates of initiation. The 5' non-coding regions of α and β -globin mRNA are 36 and 53 nucleotides long, respectively. They show few common features, making it difficult to pinpoint specific sequence differences that may be correlated with the differential rates of initiation. One significant point to arise, however, was that α -globin mRNA seemed to have a strong hairpin loop whereas β -globin mRNA did not. Thus a knowledge of the 5' non-coding sequence has not established the cause of the differential rate of initiation but has suggested that it might be caused by the differences in secondary structure.

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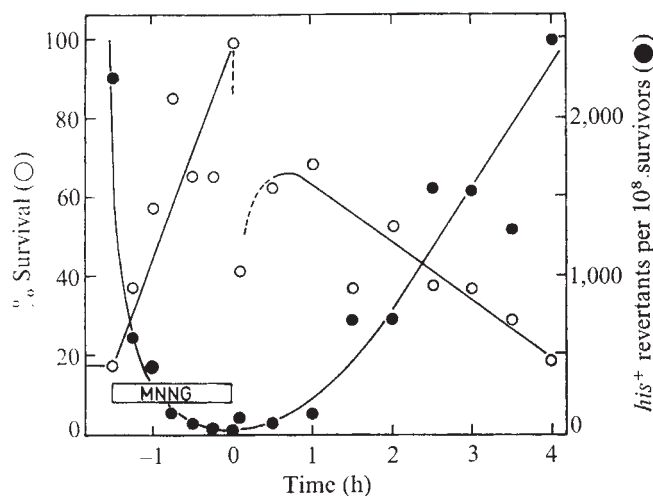
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A new pathway for DNA repair in *Escherichia coli*

MOST studies on mutagenesis involve exposing a growing population of cells to a large dose of mutagen for a short period. In a natural environment, however, cells are probably exposed more often to a low concentration of mutagens for long periods. We therefore measured the accumulation of mutants in *Escherichia coli* cells growing continuously in the presence of very low concentrations of mutagens. Because many mutagens are highly unstable, it was necessary to devise a system in which cells could be continuously exposed to fresh mutagen. To do this we took advantage of the fact that certain bacteria can grow and divide while attached to the underside of a Millipore filter. This system was originally developed by Helmstetter and Cummings¹ as a means of producing synchronous cells, because as growth medium is passed through the filter it carries away with it the unattached daughter from each pair of newly divided cells. The mutagen to be studied can therefore be stored in stable conditions and only mixed with the growth medium immediately before being passed through the filter; for example the mutagen *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG) can be stored in citrate buffer at pH 5.0 in which it has a half life of 40 h compared with 2.3 h in synthetic minimal media^{2,3}. We had intended to determine the relationship between ambient concentration of various mutagens and resulting mutation rate. The first mutagen studied was MNNG and the results were totally unexpected.

Fig. 1 The effect of previous growth in 1 $\mu\text{g ml}^{-1}$ MNNG on mutagenesis and killing by a short exposure to 100 $\mu\text{g ml}^{-1}$ MNNG. *E. coli* F26 (B/r., *thy* *his*) was grown at 37 °C with aeration to a density of 1×10^8 cells per ml in M63 medium⁶ supplemented with glucose (0.1% w/v), thymine (4 $\mu\text{g ml}^{-1}$) and histidine (40 $\mu\text{g ml}^{-1}$). At -1.5 h, MNNG (1 $\mu\text{g ml}^{-1}$) was added and samples were removed every 15 min till time zero; during this period the viable count increased 2.3-fold. At time zero, the culture was filtered and resuspended in the absence of MNNG, after which samples were removed every 30 min for a further 4 h. MNNG (100 $\mu\text{g ml}^{-1}$) was added to each sample for 5 min and the samples were then filtered, diluted and plated on nutrient broth plates and on M9 (ref. 7) plates containing thymine but not histidine. Survival (○) and *his*⁺ reversion (●) were measured. (The transient drop in survival of cells at time zero, indicated by interrupted lines, is apparently due to a transient increased susceptibility of cells to MNNG immediately after filtration.)



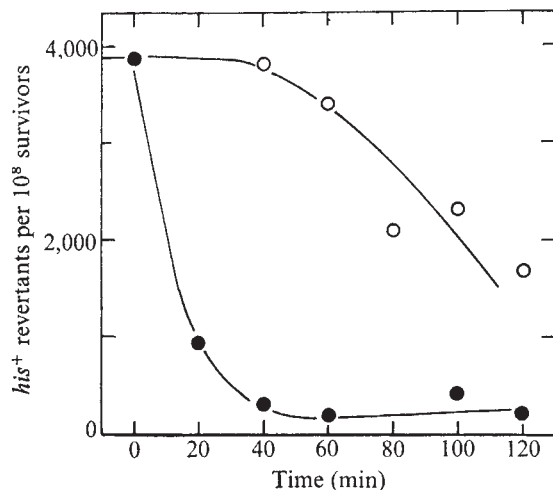
Bacteria on a filter could be kept growing for at least a week in a continuously replenished supply of medium containing $1 \mu\text{g ml}^{-1}$ fresh MNNG, and this produced a significant increase in frequency of reversion of a *his* nonsense mutation. The mutations arose only during the first hour of exposure to MNNG, however, and no further increase in frequency could be detected during the remaining 4–7 d of the experiment. (The technical details of these long term experiments will be published elsewhere and are not relevant here.) A similar result had been reported by Jiménez-Sánchez and Cerdá-Olmedo³ but they were more interested in where the mutations arose in the chromosome than in when they appeared during MNNG treatment, and so did not pursue the matter further. The simplest interpretation for our failure to collect an increasing number of mutants with time would be that the constant exposure to MNNG allows each bacterium to handle MNNG more efficiently and so become resistant to MNNG mutagenesis. Here we show that bacteria can indeed quickly become resistant to MNNG, apparently through the induction of a novel form of DNA repair.

The first and simplest experiment was to add $1 \mu\text{g ml}^{-1}$ MNNG to an exponentially growing liquid culture of *E. coli*. At various times thereafter samples from the culture were subjected to $100 \mu\text{g ml}^{-1}$ MNNG for 5 min and the resulting number of mutants and extent of killing were measured. The results clearly indicate that the bacteria had become increasingly difficult both to kill and to mutate during their growth in $1 \mu\text{g ml}^{-1}$ MNNG. On removal of the MNNG from the growing culture these properties decayed over a period of 4 h (Fig. 1). This effect looks very much like the induction and decay of a gene product and indeed, as Fig. 2 shows, the attainment of MNNG resistance did not occur in the presence of chloramphenicol and hence requires *de novo* protein synthesis.

Although we have not carried out any very extensive study of other mutagens, preliminary experiments have shown that a low level of MNNG induces resistance not only to MNNG itself but also to *N*-methyl-*N*-nitrosourea and ethyl methanesulphonate; it does not, however, induce resistance to ultraviolet light or methyl methanesulphonate.

The chloramphenicol-sensitive step that brings resistance to MNNG could be the synthesis of a protein which operates in one of two ways. The protein could prevent lesions being formed in the cells, for example, by making

Fig. 2 The effect of chloramphenicol on the induction of MNNG resistance. At time zero MNNG ($1 \mu\text{g ml}^{-1}$) was added to two cultures of *E. coli* F26. Samples were removed every 20 min and challenged with $100 \mu\text{g ml}^{-1}$ MNNG for 5 min, and the number of induced mutations was measured. One culture received chloramphenicol ($100 \mu\text{g ml}^{-1}$) for the first 20 min (○) and the other did not (●). A control experiment had previously shown that such exposure to chloramphenicol had, on its own, no effect on the response to challenge with MNNG.



the cell impermeable to MNNG or by breaking down MNNG as it enters the cell; or it could allow cells to handle the lesions more efficiently, for example, by carrying out some form of DNA repair like the inducible SOS repair system described by Witkin⁴ and George *et al.*⁵

All the available evidence seems to suggest that the effect is due to the induction of a new pathway for DNA repair, and is not due to something that prevents lesions being formed. For example, preliminary experiments have shown that methylation of DNA by ³H-MNNG occurs at approximately the same rate in induced and non-induced cells (P. Jeggo, personal communication). In addition, the survival of transforming plasmid DNA (ref. 8) that has been alkylated by MNNG *in vitro* is greater if it is assayed in cells that have been induced by MNNG (Fig. 3). Thus the

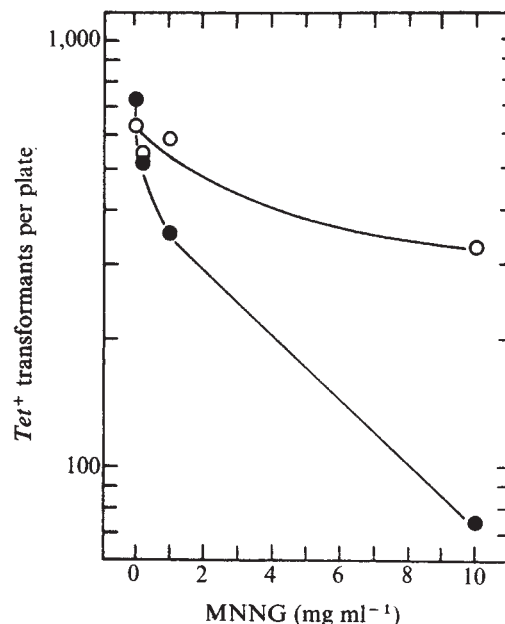


Fig. 3 The ability of transforming DNA, previously alkylated by MNNG *in vitro*, to transform induced and non-induced *E. coli* C803. MNNG was added to samples of tetracycline-resistant (Tet^r) PMB9 DNA (ref. 8) to 0.1, 1 and 10 mg ml⁻¹ at 37 °C for 25 min, and subsequently removed by dialysis overnight. These DNA samples were used to transform competent *E. coli* C803 cells (*gal*, *met*, *supII*, *hsr*⁻, *hsm*⁻, *supIII*⁺) which were either induced (○) or non-induced (●) by 1 h of growth in $1 \mu\text{g ml}^{-1}$ MNNG. The efficiency of transformation was measured by the number of Tet^r transformants produced when competent cells were transformed by $1 \mu\text{g ml}^{-1}$ DNA.

function of the induced protein(s) is probably to repair damage caused by the mutagen. But, unlike the SOS repair system^{4,5} which allows increased survival at the cost of an increased mutation rate, this system lowers the mutation rate; further, preliminary experiments have shown that it involves a different set of gene products than SOS repair (P. Schendel, personal communication). We therefore believe that these results indicate the presence in *E. coli* of a DNA repair system, which is inducible but relatively error-free.

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Wide ranging plasmid bearing the *Pseudomonas aeruginosa* tryptophan synthase genes

THE wide ranging group of plasmids comprising the P compatibility class in *Escherichia coli* (ref. 1) (the P-1 class in *Pseudomonas aeruginosa* (ref. 2)) contains within it some members capable of serving as sex factors for the transmission of chromosomal markers from one *P. aeruginosa* strain to another. Haas and Holloway³ selected variants of one such P-1 plasmid, R68, that had become highly efficient in *Pseudomonas* chromosomal gene mobilisation. Some of these variants are also efficient sex factors in *E. coli* and *Rhizobium leguminosarum*⁴. Previous work with P-1 plasmids showed that they can incorporate resistance markers from *Pseudomonas*-specific plasmids and transmit them to *E. coli*⁵. We report here the analogous incorporation of the chromosomal *trpAB* genes from *P. aeruginosa* into R68.44, and a study of the expression of these genes after transfer to *E. coli* K12.

The tryptophan synthetic pathway and the genes responsible for its several steps in *E. coli* and *P. aeruginosa* are shown in Fig. 1. In *Pseudomonas* the two genes encoding the α and β chains of tryptophan synthase, *trpA* and *trpB*, are widely separated from the remaining pathway genes; this gene pair is regulated through induction by the substrate, indoleglycerol phosphate, rather than repression by L-tryptophan as is the case for certain other *Pseudomonas trp* genes and the entire *E. coli trp* operon⁷. Genetic studies^{8,9} have suggested that this regulation is autogenous and that the *trpA* gene product is at least part of the repressor, but a direct test of this hypothesis has been impossible because of the lack of a suitable source of DNA bearing the *Pseudomonas trpAB* region.

PAC174, a strain of *P. aeruginosa* requiring lysine and carrying the plasmid R68.44, was grown in nutrient broth

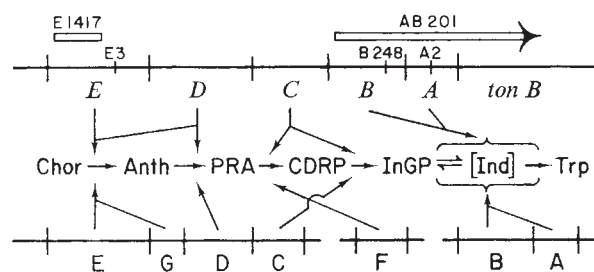


Fig. 1 Pathway of tryptophan (Trp) synthesis from chorismate (Chor). Intermediates: anthranilate (Anth), phosphoribosyl anthranilate (PRA), 1-*o*-carboxyphenylaminodeoxyribulose-5-phosphate (CDRP), indole-3-glycerol phosphate (InGP) and indole (Ind)[enzyme-bound]. Above the reactions the genes of the tryptophan operon of *E. coli* are drawn, arrows indicating the reactions their protein products participate in. Locations of the three *trp* mutations in strain IC762 are shown, along with the deletions ($\Delta trpE1417$ and $\Delta tonBtrpAB201$) present in a double deletion strain in which a set of plasmid variants similar to those in Table 1 were also obtained. Because of poorer growth yields associated with the *tonB* character, enzymic analysis was performed only in the IC762 background. Below the reactions are shown the chromosomal *trp* gene distributions for *P. putida* and *P. aeruginosa*. (The location of the *trpG* gene is deduced from indirect evidence, since mutations affecting its gene product have not yet been reported.) Arrows indicate the reactions each gene product catalyses.

together with *E. coli* W3110 resistant to rifampicin and carrying *trpA33*, a non-reverting mutation which produces an ineffective tryptophan synthase α chain¹⁰. After incubation at 37 °C for 3 d, the mixture was plated on minimal lactose agar with 50 $\mu\text{g ml}^{-1}$ of rifampicin. About one cell in 10^{10} formed a colony. All seemed indistinguishable and one clone, designated W3110*trpA33rif^R* (*ptrpAB*), was chosen for study.

It was a typical *E. coli* K12 with the antibiotic resistances and phage sensitivities characteristic of a strain carrying R68.44 (ref. 3). When R751 (ref. 11), a plasmid incompatible with R68.44, was introduced into W3110*trpA33rif^R* (*ptrpAB*), the drug resistances and the ability to grow without added tryptophan were lost. When *ptrpAB* was transferred to *E. coli* K12 strains carrying various *trp*⁻ mutations, it conferred prototrophy on those strains with defective *trpA* or *B* genes or with an extensive deletion involving these genes (Fig. 1) but not on those with mutations in *trpC*, *D*, or *E*.

Measurement of the molecular weight of covalently closed circular (CCC) plasmid DNA by sedimentation through a neutral sucrose gradient gave a value of 37.6×10^6 for R68.44 and 113×10^6 for *ptrpAB*. Thus, *ptrpAB* seems to carry at least 75×10^6 of DNA from the *P. aeruginosa* chromosome. Since the molecular weight of entire *P. aeruginosa* chromosome is about $2,100 \times 10^6$ (ref. 12), *ptrpAB* carries about 3.6% of it. *ptrpAB* is unstable; plasmid species conferring resistance to ampicillin, tetracycline and kanamycin and sensitivity to phages specific to P plasmids, but not containing the *trpAB* genes, can be transferred from *E. coli* (*ptrpAB*). Neutral sucrose gradient analysis of ³H-labelled *ptrpAB* CCC DNA (Fig. 2) shows,

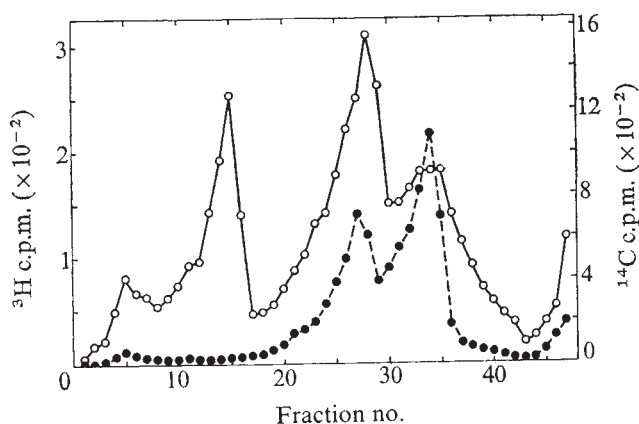


Fig. 2 Neutral sucrose gradient analysis of *ptrpAB* DNA. Covalently closed circular tertiary DNA forms of ³H-labelled *ptrpAB* and ¹⁴C-labelled R68.44 were isolated using caesium chloride-ethidium bromide density gradient centrifugation¹⁶. A mixture of the two plasmid DNAs was analysed by sedimentation through a 4.6-ml 5-20% sucrose gradient¹⁷ for 70 min at 100,000g and 20 °C. Fractions (0.1 ml) were collected directly on to glass fibre disks and, after drying, washing and redrying, were assayed for radioactivity. Sedimentation was from right to left. ○, ³H; ●, ¹⁴C. The molecular weights of the plasmid species were calculated as described¹⁷.

in addition to CCC DNA of molecular weight 111×10^6 (fraction 15), a CCC DNA species of molecular weight similar to the CCC DNA of ¹⁴C-labelled R68.44 (fraction 28). The ¹⁴C-DNA peak at fraction 34 is open circular DNA of R68.44 formed by spontaneous single-strand scission of the CCC DNA. The ³H-DNA co-sedimenting with this peak probably represents the open circular form of the smaller DNA species found in *ptrpAB* DNA.

To study the expression of the plasmid-borne *trpA* and *trpB* genes we transferred *ptrpAB* into *E. coli* IC762, a derivative of W3110 that is *his⁻*, *str^R*, and bears three

mutations in the *trp* operon, *trpE3* (a mis-sense mutation inactivating the first tryptophan-specific enzyme), *trpB248* and *trpA2* (mis-sense and ochre mutation affecting the β and α subunits of tryptophan synthase, respectively). The intact *trpD* and *trpC* genes in this triple mutant allow anthranilate taken up from the medium to be converted to indoleglycerol phosphate, but no further along the pathway (Fig. 1). IC762 cells containing *ptrpAB* have the expected antibiotic resistance complement and require histidine and tryptophan. When provided with anthranilate in place of tryptophan they grow very slowly (>10 h generation time), although their copious excretion of ferric chloride-positive material indicates that availability of indoleglycerol phosphate cannot be limiting the growth rate. Extracts of cells grown in the presence of anthranilate have extremely low tryptophan synthase activity (Table 1). (For comparison, *P. aeruginosa* PAC21, the plasmid-free parent of PAC174 and hence donor of the *trp* genes on *ptrpAB*, grown in minimal medium has 0.70 tryptophan synthase units per mg protein.)

Table 1 Tryptophan synthase levels in cells harbouring R68.44 *ptrpAB* plasmids

Strain	Tryptophan	Culture conditions	
		Anthranilate	Indole
IC762	< 0.02	No growth	No growth
IC762(<i>ptrpAB</i>)	< 0.02	0.0	No growth
IC762(<i>ptrpAB-1</i>)	< 0.02	0.70	No growth
IC762(<i>ptrpAB-2</i>)	0.02	1.42	No growth
IC762(<i>ptrpAB-3</i>)	2.57	2.09	2.07
IC762(<i>ptrpAB-4</i>)	0.08	0.83*	0.72

Strain IC762 is an F⁺*his*⁻*str*^R derivative of the triple mutant *trpE3 trpB248 trpA2* constructed and characterised earlier¹⁴. Plasmid-bearing cells were grown 16–20 h at 37 °C with shaking in 1 l of minimal medium¹⁵ containing 0.2% glucose, 0.05% acid hydrolysed casein, and 4 mg of tryptophan or 10 mg of anthranilate or indole. Reversion controls showed the strains' growth properties to be unchanged at the time of collection. Cells were centrifuged, washed once in 0.1 M potassium phosphate, pH 7.5, suspended in 5 ml of the same buffer and disrupted by sonication. Extracts were assayed for tryptophan synthase (indole → tryptophan) and protein as described^{7,8}. Specific activity is given in units (0.1 μ mol per 20 min) per mg protein.

*This culture also contained 4 mg of tryptophan because the cells cannot grow on anthranilate alone.

We have not succeeded in isolating single-step variants of IC762 (*ptrpAB*) able to grow rapidly on anthranilate. After several serial transfers of 10-ml cultures in minimal medium containing anthranilate in place of tryptophan, however, we obtained a succession of faster growing strains whose improvements were plasmid-associated. Table 1 presents enzyme assays of two members of such a series, *ptrpAB-1* and *ptrpAB-2*. Growth rates conferred by these plasmids on several *E. coli* strains lacking tryptophan synthase are about proportional to the enzyme levels; that of IC762 (*ptrpAB-2*) growing on anthranilate is approximately 4 h. Enzyme levels are quite low in cells grown in the absence of anthranilate (Table 1) and the level of enzyme induced by anthranilate is not influenced by excess tryptophan (data not shown).

Strains lacking anthranilate synthase and having *ptrpAB* as their sole source of tryptophan synthase do not grow at all on low levels (5 μ g ml⁻¹) of indole in place of tryptophan. Even those containing *ptrpAB-2* do poorly (generation time >12 h). In this they resemble anthranilate synthase-less mutants of *P. putida*⁷ and *P. aeruginosa*¹³. We sought mutants of IC762 (*ptrpAB*) able to utilise indole at this concentration, for the *Pseudomonas* precedent^{7,8} suggests that such mutants may either be constitutive for tryptophan synthase or have mutations in *trpA* abolishing the ability to grow on anthranilate but permitting indole to act

as an inducer. Indole utilisers of both types were found (Table 1). In each case the regulatory change was shown to be plasmid-associated when the extrachromosomal element was transferred to other *E. coli* strains.

The tryptophan synthase produced by *ptrpAB-1*, *ptrpAB-2* and the regulatory mutants *ptrpAB-3* and *ptrpAB-4* was found to resemble that of *P. putida* and *P. aeruginosa* in its response to monovalent cations^{7,13} and was effectively neutralised by antiserum prepared to the β_2 subunit of the *P. putida* enzyme but poorly by antiserum to the *E. coli* β_2 subunit. One millilitre of antiserum against the β_2 subunit of *P. putida* tryptophan synthase neutralises 18 units of *ptrpAB-2* enzyme produced in IC762 or the double deletion background, and 36 units of PAC21 tryptophan synthase. Antiserum against *E. coli* β_2 subunit (homologous neutralisation 1,430 units ml⁻¹) gave only 4 units ml⁻¹ neutralisation against the plasmid-derived or the PAC21 enzyme. Clearly enzyme produced in *E. coli* by the plasmid genes is very similar to that of the *P. aeruginosa* parent, though more precise characterisation awaits its availability in greater quantity and purity.

We conclude that *ptrpAB* contains, in addition to the genes of R68.44, a rather large segment of the *P. aeruginosa* chromosome including the *trpAB* gene pair. The regulation of this gene pair in *E. coli* cytoplasm seems to be similar to that in *Pseudomonas* rather than the coliforms. Any gene products other than tryptophan synthase required for this regulation must be present on the 75 $\times 10^6$ segment of presumably contiguous chromosomal DNA carried by the plasmid. As first isolated, the plasmid's *trpAB* gene activity was low. Variants were found having more normal activity levels. The nature of these variants is not clear, but the existence of an impediment to the expression of certain *Pseudomonas* genes in *Escherichia* ought to be borne in mind when intergeneric recombinant DNA experiments are contemplated.

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Phonons and the elastic moduli of collagen and muscle

BIOLOGICAL fibres make a suitable subject for the development of the science of biomaterials. The amino-acid sequence of the collagen molecule is known^{1,2}, and has been used to account for the self-assembly of collagen into native fibrils¹ and polymorphic forms³, the pattern of bands due to positive staining in electron micrographs⁴ and the low-angle meridional X-ray diffraction pattern⁵. The collagen structure to amino acid resolution in axial projection is thus completely understood. Here we relate this structural picture to the mechanical properties (and since we are dealing with a biological fibre, these are also the biological properties) of the fibres. This can be done by investigating the vibrational and other motions of the molecular assembly in collagen. The force constants of hydrogen bonds in some polypeptides have been estimated on the basis of models⁶ but no experimental measure of their magnitude has been reported. We describe measurements of the inelastic light scattering (Brillouin) spectra of collagen from rat tail tendon from which we obtain elastic moduli of the tropocollagen molecule and an estimate of hydrogen bond force constants. We have also studied effects of hydration on the spectra to obtain information about the association of water with collagen in the native state. For comparison we have made preliminary measurements on molluscan 'catch' muscle in which the polypeptide conformation is different from collagen.

Samples of collagen were excised from tail tendons of adult rats and a bundle of fibrils ~0.2 mm in diameter mounted under tension sufficient to remove the characteristic banded appearance under the polarising microscope and produce alignment of the fibrils. The Brillouin spectra were recorded using a triple-pass Fabry-Perot interferometer (based on Burleigh Instruments model DAS-1/RC140) and were excited using between 5–50 mW of argon laser light at 514.5 or 488.0 nm. The incident and scattered beams were in the horizontal plane at 90° to one another and the sample orientated at 45° to these directions such that momentum transfer was along the fibre axis. In this configuration the wavevector transfer to the sample is independent of its refractive index and is equal to $2\sqrt{2}|k_0|$ where k is the wavevector of the laser light *in vacuo*.

Figure 1 shows spectra of a sample mounted in air at 12% relative humidity at 25 °C. There is a central feature due to elastically scattered light at the laser frequency and two inelastic components due to energy gain and energy loss. The

Fig. 1 Brillouin spectra for rat tail collagen fibres at 25 °C and 12% relative humidity showing the mode polarisation.

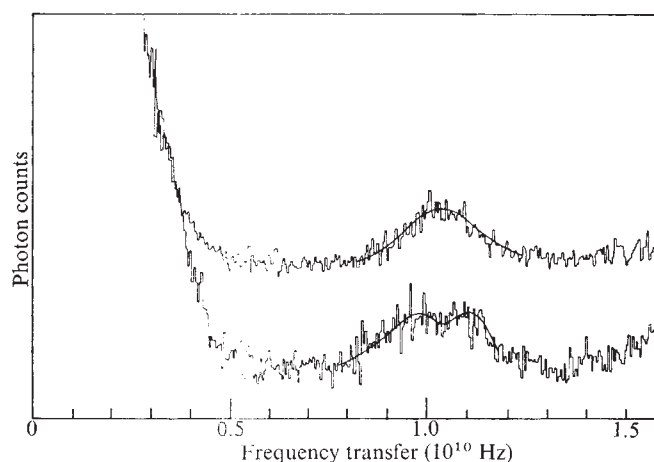
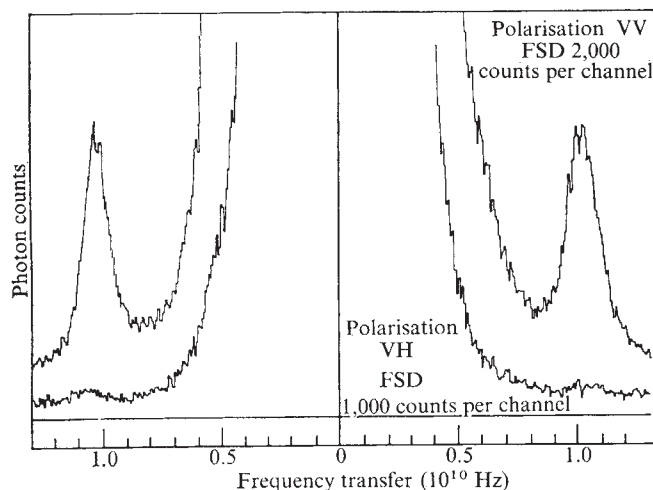


Fig. 2 Brillouin spectra from the air-dried anterior byssus retractor muscle of *Mytilus edulis* (at 25 °C) at two values of the relative orientation of the fibre and the scattered momentum transfer, q . Upper trace, $q//$ fibre axis; lower trace, q 10° off axis.

frequency shift of these components varies linearly with laser frequency indicating that they correspond to acoustic phonons. The spectra in Fig. 1 are for two different polarisations of the incident and scattered light; VV refers to both polarisations vertical, that is, perpendicular to the scattering plane, and VH refers to incident polarisation vertical and scattered polarisation horizontal. The Brillouin components are of order 40-times stronger in VV than in VH polarisation; we can thus identify the scattering as due to longitudinal acoustic waves propagating parallel to the fibre axis⁷. No transverse acoustic modes have been observed at frequency shifts greater than 6.0 GHz despite an extensive search.

Figure 2 shows spectra from the anterior byssus retractor muscle of *Mytilus edulis*. The specimen had been dried in air at 25 °C and was mounted in the same manner as the dry collagen sample. Only one side of the elastic peak is shown; in the upper spectrum the sample is at exactly 45° to the incident laser beam and, by coincidence, Stokes and anti-Stokes Brillouin components from adjacent orders of interference overlap. In the lower spectrum the sample is tilted 20° towards k_0 such that wavevector transfer is about 10° from the fibre axis and the two Brillouin components are then just resolved because of the anisotropy in the acoustic velocity.

Figure 3 shows spectra for samples of collagen in 0.15 M NaCl solution; collagen in air at 100% relative humidity and for pure water in an equivalent scattering geometry. The measurement in 0.15 M NaCl was made with the fibre orientated as before but pressed between microscope slides, thereby ensuring that the fibre was bathed continually in the solution by capillary action. This arrangement prevents drying and gives the closest approach to the native condition of the collagen fibre (A. M. and B. B. Doyle, unpublished). For the measurement at 100% relative humidity the fibre was mounted in a cell at 25 °C through which humidified air was circulated for 2 d before the measurements started.

Figure 4 illustrates the result of experiments in which the collagen fibre was held in air at 25 °C at various relative humidities. The frequency of the longitudinal acoustic mode decreases as the humidity is increased and for high humidities approaches the frequency observed in 0.15 M NaCl (compare upper two spectra in Fig. 3). The intensity scales were different for each spectrum. In fact the Brillouin intensity is more or less unchanged over the whole range of humidities. The peak widths were instrumental. Spectra at different humidities were difficult to reproduce for two reasons. First, local heating by the laser beam dried the sample locally and thus the observed frequencies were power dependent; the spectra of Fig. 4 were obtained

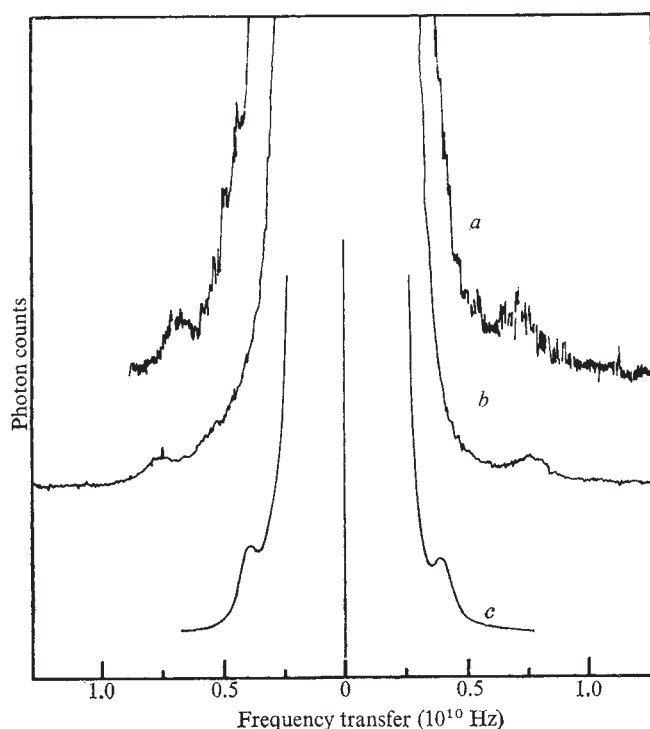


Fig. 3 Brillouin spectra from collagen *a* in 0.15 M NaCl solution between slides and *b* in 100% relative humidity vapour compared with *c*, the Brillouin spectrum of water. Brillouin scattering is from rat tail collagen laser heating effects.

using the lowest possible laser power (~ 5 mW) but even so at 84% and 100% humidity are appreciably affected by local drying, as can be seen by reference to Fig. 3. Second, hysteresis in the hydration of the fibres was observed; the frequency depended on whether a previously dried fibre was humidified or a wet fibre dried to a particular humidity. Experiments were performed to see if the Brillouin frequency of 84% humidity depended on external load attached to the fibre. The frequency was the same, within experimental error, for tensions between 1×10^7 and 4×10^8 dyn cm^{-2} .

Table 1 gives the measured longitudinal mode frequencies, corresponding acoustic velocities and elastic moduli, in various conditions. The calculation of elastic modulus requires knowledge of the density of the material. For dry collagen and muscle we estimate the density ρ from the excluded volumes of different amino acid residues present⁸ and their masses. For dry collagen $\rho = 1.43$ g cm^{-3} and for muscle $\rho = 1.40$ g cm^{-3} .

In the native state, assumed to be at 95% humidity, $\rho = 1.16$ g cm^{-3} (ref. 1) (assuming density of water = 1) for collagen. The amount of water present was taken from the adsorption isotherms for collagen¹⁰.

To discuss the sound velocities and moduli in collagen and muscle we take as a basis the calculations of Fanconi and Peticolas⁶ on poly-L-alanine. The paramyosin chain of the muscle, where the α helix is gently supercoiled with a second molecule, 3.5 residues per turn (1.485 Å repeat distance) as opposed to 3.6 in the α helix (1.5 Å repeat distance), is closely related to this synthetic α -helical polymer. The acoustic slope of the seven mass model poly-L-alanine can be fitted to the observed frequency and the calculated wavevector when one chooses a hydrogen bond force constant of about 0.07 m dyn $\text{\AA}^{-1}$. For synthetic macromolecules¹¹ the modulus decreases strongly with degree of coiling because bond twisting rather than bond stretching or angle opening strains accommodate the stress. This is presumably also the case for the polypeptide and so this hydrogen bonding force constant must, for the moment, be regarded as a fitting parameter. By the same principles, the higher modulus of collagen compared with muscle is consistent with the collagen conformation being more extended (3.3 residues per turn and 2.86 Å between residues). The moduli obtained by light scattering are about two orders of magnitude stiffer than the values derived from static measurements involving macroscopic extensions. This is again a familiar observation for synthetic polymers¹¹ and indicates inhomogeneous strain for measurements.

The sound velocity in collagen decreases monotonically with the degree of hydration (Table 1). Since the bulk density decreases on hydration it is supposed that both softening of the intrachain forces, particularly the hydrogen bonds, and rigid loading of the chains by the adsorbed water occurs. At 85% humidity there is about one water molecule adsorbed per peptide on the average¹⁰. At this point the mass per unit length of the chain has increased by 35% giving a frequency decrease of 17% compared with the 32% actually found. The decrease can be parameterised by first accounting for the decrease in sound velocity due to rigid loading, and then estimating the changes in H-bond force constant by interpolating the reduced sound velocities between values derived from the seven atom model calculations of Fanconi and Peticolas⁶ with a range of assumed H-bond force constants. This procedure gives the figures in the last column of Table 1. This approach is preferred to a discussion involving changes in the molecular conformation as a result of hydration since there is no detectable change in the high-angle X-ray diffraction pattern. At very high hydration the approach used here breaks down and the effects may be discussed using extended hydrodynamics¹².

In summary, measurements of the inelastic light scattering

Table 1 Acoustic properties of collagen and muscle at 25 °C

Substance	Q ($\text{\AA}^{-1}$)	v (GHz)	Sound velocity (cm s $^{-1}$)	Density (g cm $^{-3}$)	Elastic modulus (dyn cm $^{-2}$)	H-bond force constant (m dyn $\text{\AA}^{-1}$)
Poly-L-alanine (theory)	0.18	1,800	6.4×10^5	1.51*	6.18×10^{11}	0.3
	0.23	1,800	4.8×10^5	1.51*	3.48×10^{11}	0.15
	0.23	258	6.9×10^4	1.51*	7.2×10^9	0.0
Muscle 12% relative humidity	1.727×10^{-3}	9.69	3.53×10^5	1.40*	1.74×10^{11}	0.1†
Collagen at 0% relative humidity	1.727×10^{-3}	10.65	3.88×10^5	1.43*	2.15×10^{11}	0.11‡
Collagen at 12% relative humidity	1.727×10^{-3}	10.29	3.74×10^5	1.4†	1.96×10^{11}	0.11‡
Collagen at 30% relative humidity	1.727×10^{-3}	9.00	3.27×10^5	1.38†	1.47×10^{11}	0.09‡
Collagen in 0.15 M NaCl solution (assumed equivalent to 85%)	1.727×10^{-3}	7.20	2.62×10^5	1.31†	9.0×10^{10}	0.08‡
Collagen (air dried) ¹³		(a) Macroscopic stress-strain measurements			$\sim 10^9$	
		(b) Using the 660-Å reflection to measure strain			$\sim 10^9$	
		(c) Using the 2.8-Å reflection to measure strain			$\sim 2 \times 10^9$	
Collagen (dry) ¹⁴		X-ray measurements			1.22×10^{10}	

*Assumed 0% humidity and density determined from peptide molecular weights and exclusion volumes¹⁵ from Chothia.

†Calculated from the adsorption isotherms. Rougvie and Bear¹⁰ by taking arithmetic mean density.

‡By interpolation between calculated values of sound velocity from Fanconi and Peticolas⁶.

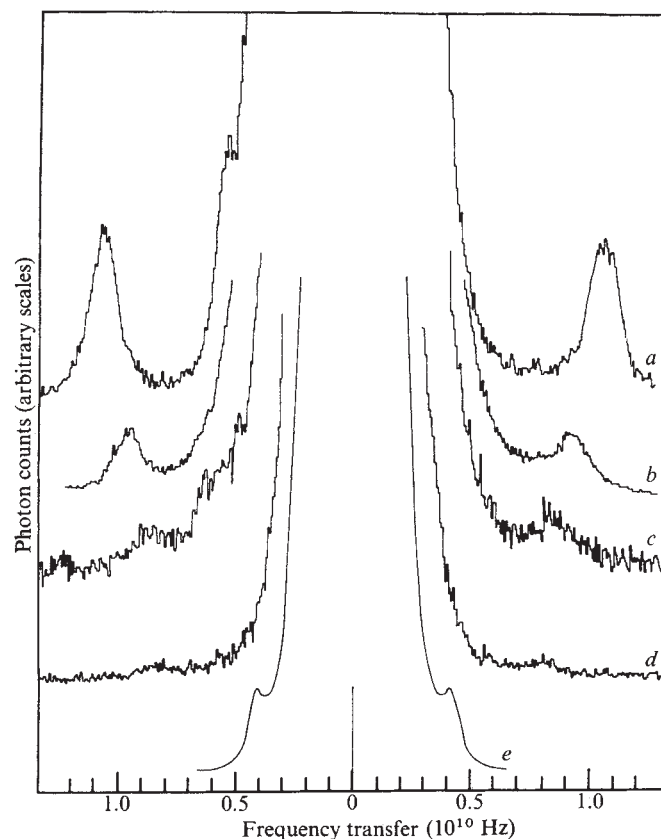


Fig. 4 Dependence of the Brillouin frequencies on degree of hydration for rat tail collagen at 25 °C. Obtained at lowest possible laser power at relative humidities of *a*, 0%; *b*, 30%; *c*, 84%; *d*, 100%. *e*, Spectrum of ordinary distilled water (not dust free).

from collagen and muscle give values for the velocities of acoustic waves and elastic moduli in the medium. For dry collagen this leads to an estimate of the hydrogen bond strength. For material in the native state the measurements are capable of giving information about the dynamics of water molecules in the material.

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Crystal structure of anhydrous cholesterol

CHOLESTEROL, the most abundant steroid in the animal kingdom, is found mainly as a component in cell membranes and lipoproteins. The presence of the hydrophilic C(3) hydroxyl group in this otherwise hydrophobic molecule allows cholesterol to occupy a position at polar–nonpolar interfaces. Crystals of 3-hydroxy steroids and their hydrates show a tendency towards double layer structures with an end-for-end arrangement of approximately parallel molecules¹. As this molecular organisation is generally similar to that in membranes, it can be expected that some of the principles of intermolecular association in membranes might be revealed in the molecular packing in the crystal structures. Another common feature in these structures is the presence of more than one molecule in the crystallographic asymmetric unit. While this is a complicating factor in structure analysis, it clearly increases the amount of information regarding preferred conformation and intermolecular association to be gained from a structure determination. Craven² has recently reported the remarkable crystal structure of one form of cholesterol monohydrate. The arrangement of the eight crystallographically independent molecules in the triclinic cell shows unusual local pseudo-symmetry. One pair of molecules is related to another by a non-crystallographic $b/2$ translation, while a third pair, oriented end-for-end relative to the first, is related to the fourth by a similar $a/2$ translation. Non-crystallographic twofold rotation symmetry relates members of the first and the third pairs. The overall structure is a stacking of bilayers of 33.9 Å total thickness. We report here that anhydrous cholesterol crystals are also triclinic, with eight independent molecules per cell. This structure offers additional insight into the packing of cholesterol molecules, and a comparison with the monohydrate structure.

Colourless lath-shaped crystals were obtained by cooling a saturated acetone solution of cholesterol. The cell dimensions are $a=14.172(7)$, $b=34.209(18)$, $c=10.481(5)$ Å, $\alpha=94.64(4)$, $\beta=90.67(4)$ and $\gamma=96.32(4)^\circ$, in agreement with values reported by Bernal *et al.*¹, and converted to a reduced cell³: $a=14.00$, $b=33.71$, $c=10.46$ Å, $\alpha=94.5^\circ$, $\beta=90.0^\circ$ and $\gamma=95.9^\circ$. Since cholesterol is an optically active compound, the space group of the triclinic crystals is $P1$. Each unit cell contains eight $C_{27}H_{46}O$ molecules; thus there are 224 non-hydrogen atoms per asymmetric unit. The calculated density is 1.021 g cm^{-3} ; the experimental value (floatation) is 1.03 g cm^{-3} . A total of 10,399 unique reflections were measured to the limit $2\theta(\text{CuK}\alpha)=100^\circ$ using a Syntex P1 diffractometer with a graphite monochromator.

The structure was solved by a combination of vector search methods⁴ and direct methods⁵. Vectors representing the known geometry of the rigid 21-atom 3-hydroxy steroid group were used in Patterson-space orientation searches which gave several probable orientations of this group in the crystal. Vectors between steroid groups in these orientations were used in translation searches. In this way it was possible to position three oriented steroid groups in relation to each other in such a way that searches with the three sets of inter-group vectors gave a consistent result. This 63-atom partial structure was used to calculate input

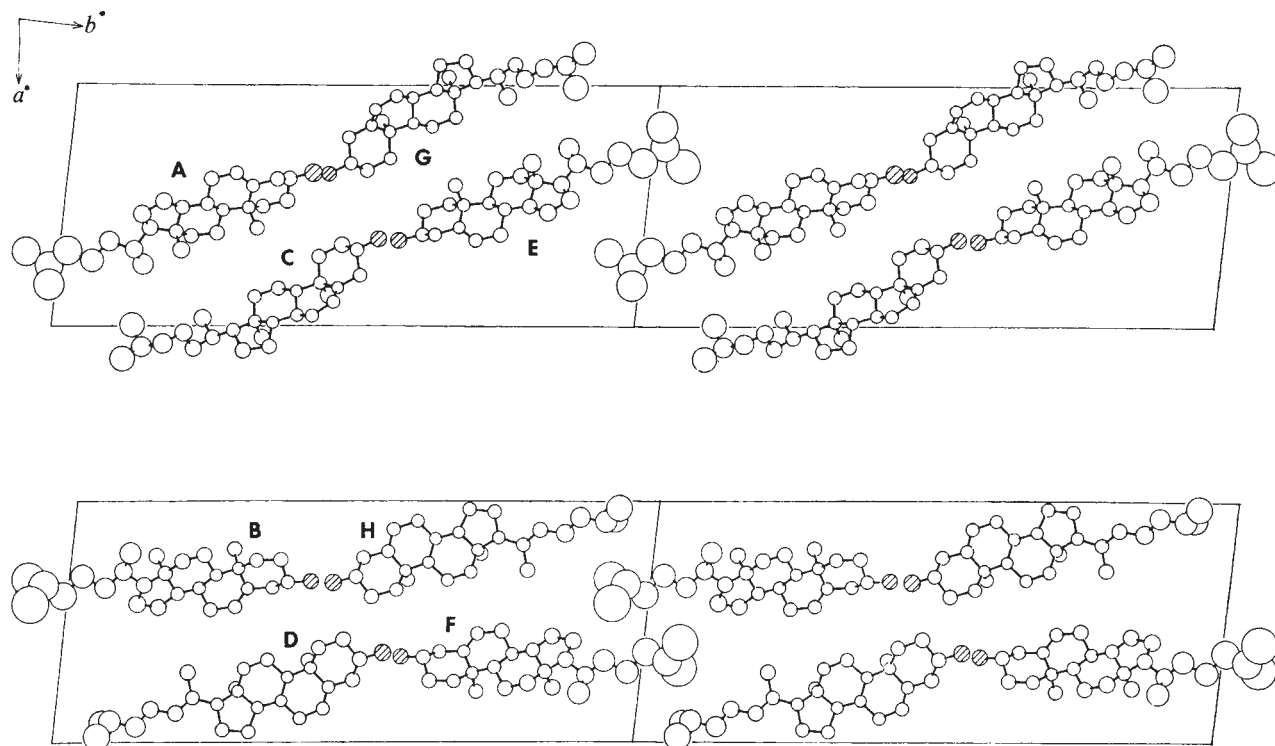


Fig. 1 The crystal structure of anhydrous cholesterol viewed along c (out of page). The upper figure is to be superposed on the lower one to give the complete structure. Two unit cells ($x = 0-1$; $y = 0-2$) are shown, displaying non-crystallographic twofold symmetry axes at $x \approx 0.5$; $y \approx 0.5, 1.0$ and 1.5 . Atoms are shown as 50% probability thermal motion spheres. Oxygen atoms shaded.

phases to the MULTAN programme³, modified to hold selected input phases fixed throughout the phase extension and refinement. The structure then emerged in a straightforward manner. About 80% of the hydrogen atoms were found in difference Fouriers, including those eight which are involved in hydrogen bonding. Refinement by block-diagonal least squares, with anisotropic C and O thermal parameters, gave $R=0.056$ for 8032 reflections with $I > 2.33\sigma(I)$.

There are no significant differences between the steroid skeletons of the eight molecules in this structure, the

average atom-atom misfit in superposing steroid skeletons being 0.07 Å. The conformation of the tail part of the cholesterol molecule shows considerable variation, however, as shown in Fig. 1, where molecules A and C represent two extreme cases in their C(17)–C(25) distances of 6.60 and 5.51 Å, respectively. This variability in the hydrocarbon chains is less pronounced in the monohydrate² where the chains are partially folded to an approximately equal degree.

The hydrogen bonding pattern (Fig. 2) consists of two independent chains of hydrogen bonds running parallel to

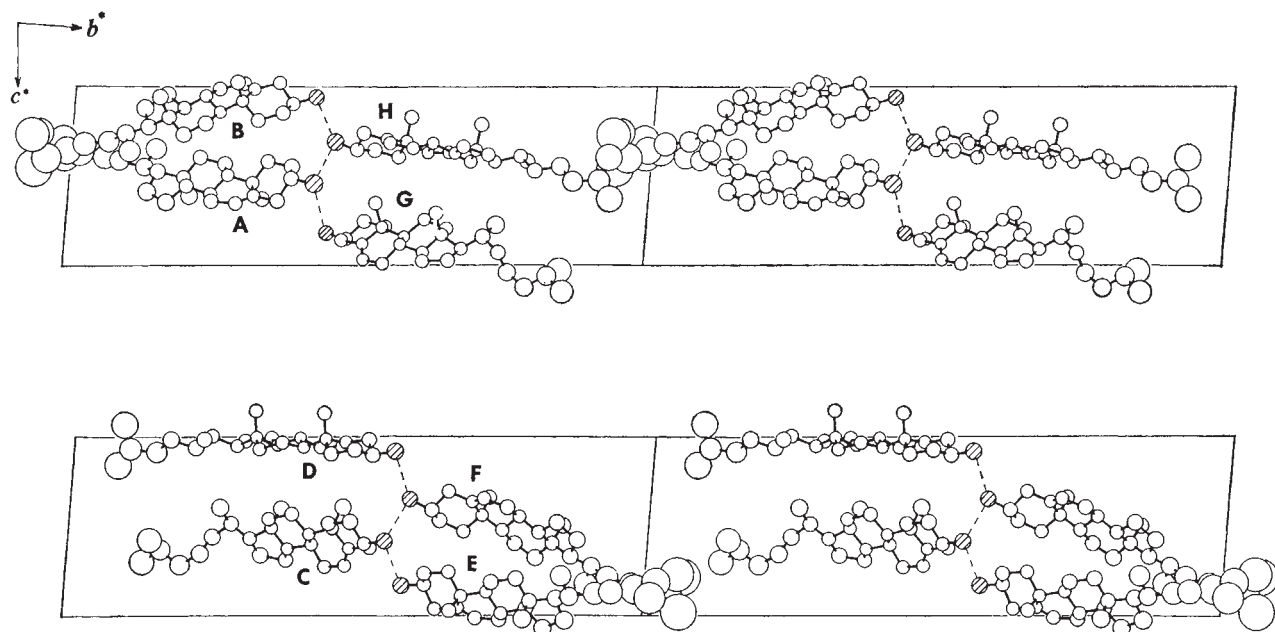


Fig. 2 The structure viewed along a (into page). Two unit cells ($y = 0-2$; $z = 0-1$) are shown, as in Fig. 1. The dashed lines indicate hydrogen bonding.

c with an average O . . . O distance of 2.87 Å. The hydrogen bond chains are staggered by ± 2.18 Å about a mean plane parallel to *ac*. This corrugated sheet of separated, parallel chains is to be compared to the pleated sheet of hydrogen bonds in the monohydrate.

The steroid planes of the eight molecules show no tendency towards parallel packing. The dihedral angles between least-squares planes of adjacent molecules range from 3° to 88°. The long molecular axes, while generally oriented along *b*, show considerable variation in direction. The angles between the least-squares lines calculated from any two molecules range from 4° to 36°. This is in contrast to the monohydrate, in which all the ring systems are approximately parallel and aligned.

Careful examination of the adjacent unit cells (Fig. 1) reveals an approximate local symmetry axis at $x=0.5$, $y=1.0$ along a direction making angles of 90.0, 94.5 and 0.7° with the crystal axes *a*, *b* and *c*, respectively. A 180°

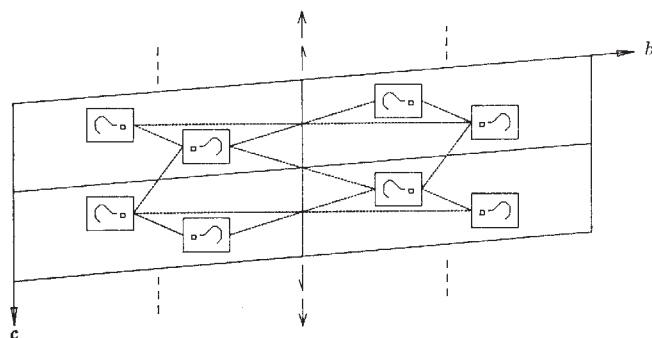


Fig. 3 Schematic representation of the non-crystallographic symmetry in four adjacent cells. The left- and right-leaning question marks represent, respectively, the molecular clusters ABCD and EFGH. Local symmetry elements shown are co-axial twofold rotation and screw axes at $y = 1$ and a 'twofold screw axis' with alternating translational components of 0.25 and 0.75 *c* at $y = 0.5$ and 1.5. This diagram applies at $x = 0$ and 0.5.

rotation about this axis brings the leftmost molecules A, B, C and D into close coincidence with the molecules E, F, G and H of the adjacent (right hand) cell. The mean atom-atom misfit between the 112 atoms of the first four molecules, turned as a rigid group, and the second 112 atoms is only 0.24 Å. This fit is as good in the C(17) chain regions as it is in the steroid skeleton regions. The remaining molecules E, F, G, H of the left hand cell are related to A, B, C, D of the right hand cell by a pseudo-twofold screw axis which is coaxial with the pseudo-twofold rotation axis. Figure 3 illustrates in diagrammatic fashion the relationships among the molecules of four adjacent unit cells. Non-crystallographic symmetry axes can also be seen to exist at $x=0.5$, $y=0.5$ and 1.5 in Fig 3. These are 'screw axes' whose translation components alternate between (very nearly) $c/4$ and $3c/4$, instead of the usual one-half cell translation.

With the presence of this pseudo-symmetry, the non-crystallographic asymmetric unit contains four molecules. These four 'unique' molecules are A, B, C and D. As can be seen in Fig. 2, there is no hydrogen bonding between these molecules. It seems that this unique structural unit is held together primarily by hydrophobic interactions.

The number of 'unique' molecules, that is, molecules not related to each other by either local or strict symmetry,

is the same in the monohydrate² and the anhydrous form. The nature of the non-crystallographic symmetry in the two structures is entirely different; there is no non-crystallographic half-cell translation in the anhydrous structure. Yet, in each case, the non-crystallographic symmetry is obeyed locally to a remarkably high degree.

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Direct evidence for an interaction of β -adrenergic blockers with the 5-HT receptor

ADVANCES in neurotransmitter receptor identification and quantification by biochemical techniques involving radio-labelled analogues of putative neurotransmitters have led to the characterisation of a number of receptor systems in mammalian brain tissue¹; these include the catecholamines^{2,3}, 5-HT⁴, glycine⁵ and GABA⁶ receptors. The development of these techniques has provided a means of screening chemical compounds for their effects on different receptor systems⁷. The central nervous system effects of ($\pm$)-propranolol (Inderal, ICI) in animals are well documented^{8,9}. This drug has therapeutic benefit in man in anxiety¹⁰, and is also reported to have similar benefit in schizophrenia^{11,12}, essential tremor¹³ and drug dependence¹⁴. The origin of these effects is not understood but theories about its mode of action include β -adrenergic blockade¹⁵, membrane stabilisation⁸ and a 5-HT receptor blocking action^{16,17}. We report here that propranolol a stereospecific affinity for the 5-HT receptor from rat brain which is similar in magnitude to the putative 5-HT receptor blocking drug methylsergide.

Specific binding of ³H-5-HT was measured by the method of Bennett and Snyder⁴. The whole brain (minus the cerebellum) of a freshly killed Alderley Park rat (250-350 g) was rapidly removed and homogenised in 30 ml of ice-cold 0.32 M sucrose with five passes of an air-driven glass-Teflon homogeniser. After centrifugation at 1,000g for 10 min, the supernatant was subjected to 17,000g for 30 min to obtain a crude mitochondrial pellet. The pellet was lysed with 30 ml of ice-cold distilled water and briefly homogenised as before. The lysate was then spun at 10,000g for 20 min. The pellet from this spin, a bilayer with a soft, buffy upper layer was washed carefully to collect the upper layer and the resultant supernatant centrifuged at 48,000g for 20 min. The pellet was suspended in 6 ml of 75 mM Tris-HCl buffer (pH 7.4) containing 25 mM MgCl₂·6H₂O and 50 μ g ml⁻¹ of nialamide.

Incubations (15 or 16.5 min, 37 °C) were carried out in glass test tubes in a 1 ml final volume containing 75 mM Tris-HCl (pH 7.4), 25 mM MgCl₂·6H₂O, 5 nM ³H-5-HT (specific activity 14 Ci mmol⁻¹, Radiochemical Centre, Amersham), 640-840 μ g of protein, the appropriate concentration of the drug under test and in the presence or absence

of 10^{-6} M 5-HT. Incubations were terminated by filtration under reduced pressure through Whatman GF/C filter papers. The filtration procedure consisted of quenching the incubation with 4 ml of ice-cold buffer, rapid filtration, followed by two further washes with 4 ml of buffer. The complete washing procedure usually took less than 10 s. The residual radioactivity on the filter papers was counted at 31% efficiency in a PCS scintillant (Amersham-Searle). Protein was estimated by the method of Lowry *et al.*¹⁸. 'Nonspecific' binding, defined as the radioactivity left on the filter paper in the presence of 10^{-6} M 5-HT, was subtracted from total binding in each experiment to give 'specific' or receptor binding. Specific binding at 5 nM ³H-5HT was in the range 55–65% of total binding.

In accord with Bennett and Snyder⁴, we have shown that the specific portion of ³H-5-HT binding is rapid, reversible, saturable and of high affinity ($K_D = 11.7 \pm 2.5 \times 10^{-8}$ M, average of three experiments, $\pm$ s.e.m.). The concentration at which specific ³H-5-HT binding is inhibited by 50% (IC_{50}) by the two putative 5-HT blocking drugs, methysergide and cyproheptadine, are given in Table 1 and are close to the previously reported values⁴. Also shown in Table 1 are IC_{50} values for the 5-HT receptor of a number of β -

Table 1 Effects of various drugs on ³H-5-HT binding to crude synaptic membranes

Drug	Half maximal inhibition of specific ³ H-5-HT binding (M)
5-HT	$1.7 \pm 0.3 \times 10^{-8}$
Methysergide	$2.8 \pm 0.3 \times 10^{-7}$
Cyproheptadine	$4.8 \pm 1.1 \times 10^{-8}$
(-)-Propranolol	$2.1 \pm 0.4 \times 10^{-7}$
(+)-Propranolol	$1.2 \pm 0.2 \times 10^{-8}$
(±)-Propranolol	$6.0 \pm 1.2 \times 10^{-7}$
(±)-Alprenolol	$3.0 \pm 0.5 \times 10^{-7}$
(±)-Oxprenolol	$4.3 \pm 0.8 \times 10^{-7}$
(±)-Pindolol	$7.9 \pm 0.8 \times 10^{-7}$
(±)-Practolol	$> 10^{-4}$
(±)-Atenolol	$> 10^{-4}$

³H-5-HT binding was assayed as described in the text. Results are means $\pm$ s.e.m. of triplicate determinations from at least three separate experiments using six different concentrations of the drugs indicated. The concentration of the drug giving half maximal inhibition of specific ³H-5-HT binding was determined by log probit analysis.

adrenergic blocking drugs. Propranolol has a substantial affinity for the 5-HT binding site, indeed (-)-propranolol is equipotent with the 5-HT blocking drug, methysergide, in displacing ³H-5-HT from its receptor. The interaction of propranolol with the 5-HT receptor is stereospecific as is evidenced by an approximately 58-fold lower affinity of the isomer (+)-propranolol, the racemate drug (±)-propranolol is about half as potent as the (-)-isomer alone. Other β -adrenergic blocking agents also have substantial affinity for the 5-HT receptor (Table) although β -blockers which are cardioselective *in vivo*, for example practolol and atenolol, have IC_{50} 's greater than 10^{-4} M.

Thus, we have demonstrated a stereospecific interaction of propranolol and other β -blockers with the 5-HT receptor, an action which was previously unknown for this class of drug. It seems that the affinity of β -blockers for the 5-HT receptor does not necessarily parallel their β -blocking potency in tissues outside the central nervous system since pindolol, an extremely potent β -blocker, is less potent in displacing ³H-5-HT than (-)-propranolol. Green and Grahame-Smith¹⁷ have suggested, on the basis of the blocking action of (-)-propranolol on a L-tryptophan induced hyperactivity syndrome in rats, that this drug inhibits central 5-HT function. The direct interaction of propranolol with

5-HT receptor reported here is in accord with these findings. The significance of these observations in relation to the effects of propranolol in the central nervous system of animals and man remains to be evaluated.

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Errata

In the article 'Possible detection of a radio event correlated with a γ -ray burst' by N. Mandolesi *et al.*, *Nature* **266**, 427–429, the legend to Fig. 2 should have read: Fig. 2a, Balloon horizon; b, Medicina horizon in equatorial coordinates . . .

In the article ' β -Endorphin *in vitro* inhibition of striatal dopamine release' by Horace H. Loh *et al.*, *Nature* **264**, 567–568, the last sentence beginning in the second column of p.567 should have read: Met⁵-enkephalin, on the other hand, did not produce a significant inhibition of tritium overflow in concentrations as high as 100 μ M. Naloxone, at an equimolar concentration, produced a significant blockade of the inhibition by morphine and β -endorphin, the blockade being more complete in the presence of morphine than in the presence of β -endorphin.

Corrigenda

In the article 'Pigmentation of the ladybird beetle *Coccinella septempunctata* by carotenoids not of plant origin' by G. Britton, W. J. S. Lockley, G. Harriman & T. W. Goodwin, *Nature* **266**, 49–50 the authors stated that phytoene, ζ -carotene and β -zeacarotene had not been detected before in any insect. Although no details were given of their characterisation, the detection of these carotenoids in certain butterflies has, however, been reported previously^{1–3}.

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In the article 'Nucleosynthesis and anomalous xenon and krypton in carbonaceous chondrites' by J. B. Blake and D. N. Schramm, *Nature* **263**, 787, the references on the following sentences with proper reference numbers should have read: Perhaps the explanation of the anomalous light isotopes lies in either the p-process^{9,10} or in fractionation⁷. If the light xenon component is due to the p-process then ¹³⁰Xe might be affected, which would change the normalisation⁹.

reviews

Birth of a Universe

John Barrow

The First Three Minutes. By Steven Weinberg. Pp. 188. (Basic Books: New York, March 1977; Andre Deutsch: London, September 1977.) \$8.95; £4.50.

DURING the twentieth century, cosmology has been transformed from metaphysics to physics, creating one of the most exciting arenas for scientific investigation. The fundamental laws of physics on the one hand and dramatic progress in observational astronomy on the other make it possible to reconstruct a general evolutionary picture of the Universe's past history with some certainty.

If one takes the present structure and reverses the sense of the observed expansion then extrapolation backwards in time reveals increasingly denser and hotter cosmic epochs. The times of important event strata can be established in the manner of geology until eventually a 'big bang' is envisaged 10,000 Myr ago. This hot scenario for cosmogeny is based on the existence of a cosmic background radiation field, predicted by Gamow in 1948, and detected at microwave-lengths by Penzias and Wilson in 1964. The photons of this radiation background are now unanimously interpreted as a vestigial remnant of the extreme conditions prevailing in the primordial fireball itself, when matter was completely ionised and the Universe a high energy physicist's paradise.

The increasing temperature as we move further into the past towards the 'bang' ensures that cosmology can provide a remarkable theoretical laboratory for particle physics. Of particular importance has been the realisation that during the first few minutes of expansion from the 'bang', physical conditions were expedient for the fusion of light nuclei having distinctive abundances. The fossilised abundances of helium and deuterium have been detected in precisely the range predicted by the simplest models and reassure us that a view into the early opaque phase of the Universe's life is possible by indirect means. The contingency of thermodynamic equilibrium in the first few seconds washes away all the uncertainties surrounding the initial state and enables laboratory physics to develop a fairly complete description of events.

Steven Weinberg has again very successfully turned his attention from the world of microphysics, where his contributions are well known, to cosmology. *The First Three Minutes* is a clear and challenging description of the Universe's early evolution suitable for the informed layman and practising scientist alike. The author has vividly explained the essential concepts and physical processes involved, without recourse to mathematics or the details of general relativity, by introducing the necessary physical ideas as his story unfolds. One of the remarkable features of the book is the manner in which the author has maintained the rigour of his physical argument throughout, never sacrificing logical continuity in the interests of speed or oversimplification.

The first three chapters are partially historical in content, describing the discoveries of the universal expansion and microwave background radiation. Unfortunately, no explanation of the crushing blow dealt to the steady-state theory by the latter observations is provided. Such a description is essential in a work intended for the non-specialist, who often believes this theory still to be in strong competition with the 'big bang' cosmologies.

In the fourth chapter the physics of black-body radiation is described with great clarity, together with the conserved quantities necessary to specify the microstructure of the Universe uniquely. This careful build-up prepares the reader for the key chapter in which the phenomena peculiar to the first three minutes of expansion from the origin are expounded frame by frame. The final chapter is an ex-

cursion into the history of science in an effort to discover the reasons for scientists' procrastination in establishing this picture when the necessary observational and theoretical ingredients were apparently available.

Criticisms of the book are minor. Perhaps a little more space could have been allocated to the first three minutes rather than the past fifty years. The opportunity could then have been taken to mention Hawking's exciting work on primordial black holes or problems surrounding the existence of a singularity in spacetime. Some more clear diagrams displaying the evolutionary phases considered and their generic phenomena would also have been welcome landmarks to establish the overall panorama more vividly. Finally, a little more space might have been devoted to the conceptual problems associated with the standard model; these latter are of great intrinsic interest and motivate much current work in cosmology. Weinberg more than atones, however, for these failings with many fascinating digressions into points of basic physics and by a lucidity that makes for compelling reading. The provision of a glossary of technical terms, detailed index and bibliography are further attractive features of what will surely become a landmark in the exposition of the subject.

This book can be strongly recommended to all as the place to begin or reinforce their education in 'pre-historic' cosmology. □

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Nerve, synapse and muscle

Electrobiology of Nerve, Synapse and Muscle. Edited by J. P. Reuben, D. P. Purpura, M. V. L. Bennett and E. R. Kandel. (Raven: New York, 1976.) \$30.

WRITTEN by his students and associates, this *Festschrift* volume in honour of Harry Grundfest contains a collection of recent studies on nerve, synapse and muscle. As might be expected from Grundfest's own interests, this volume ranges widely and provides fascinating glimpses of the ways in which an im-

portant area of biology has grown and developed. The chapters are uniformly interesting and no electrophysiologist could pick up this book and fail to learn something new. Cell physiologists around the world will be delighted that Harry Grundfest's contribution has been honoured in this way.

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Mammalian cell membranes

Mammalian Cell Membranes. Vol. 2: The Diversity of Membranes. Vol. 3: Surface Membranes of Specific Cell Types. Edited by G. A. Jamieson and D. M. Robinson. (Butterworths: London and Boston, Massachusetts, 1977.) £18 each volume.

THESE two volumes continue an extensive coverage of the structure and function of cell membranes under the general editorship of G. A. Jamieson and D. M. Robinson. As in the first volume of the series, the editors adopt a relaxed attitude towards the form and content of each contribution, with the inevitable results of an uneven mixture of styles and a considerable amount of overlap in content. Nevertheless, a considerable amount of enjoyment is to be had from these books.

In the opening chapter of volume 2, Emmelot provides an excellent short account of the composition and organisation of the plasma membrane. His discussion of the interactions of the plasma membrane with microtubules and microfilaments, one of the growth areas in present-day membrane research, is particularly useful and includes a reminder that some of the initial effects of various drugs disrupting cytoplasmic contractile elements may be directly on the plasma membrane. Kramer and his colleagues then present a personal and very interesting account of membrane flow, the migration of components from the membranes of the endoplasmic reticulum to other membranes including the plasma membrane.

These two rather general reviews are followed by short chapters concerned, respectively, with membranes of the Golgi apparatus (Favard), mitochondria (Capaldi), lysosomes (Wattiaux), microbodies or peroxisomes (Tolbert and Donaldson) and nuclei (Fry). The volume is rounded off with two somewhat dated accounts of myelin and basement membranes having no full reference later than 1973, a serious drawback in these rapidly expanding fields, in which new membrane components are turning up regularly to transform previous views of membrane organisation. It is surprising too that the primary sequence of the basic protein of myelin, which was established several years ago, is nowhere discussed, particularly in connection with its encephalitogenic properties.

The most complete analysis of glycoprotein structure is in the first chapter in volume 3 by Segrest, which is particularly valuable in presenting his current views on the molecular topography of the non-polar domain of this glycoprotein. There is interesting speculation on the state of self-association of glycoprotein within the membrane and the possibilities of these aggregates in facilitating transport. One of the editors (Jamieson) provides an authoritative summary of platelet ultrastructure, perhaps the clearest case for intimate inter-relationships of the plasma membrane with underlying microtubules in a cellular function. Jamieson also summarises the intriguing evidence for collagen-platelet interactions being mediated through surface located glycosyl transferase. As Jamieson points out, some of the disagreement in this area may be due to an unwarranted extension of his original theory, concerned with simple adhesion of platelets on to collagen, to collagen-induced platelet aggregation. The membrane systems of lymphocytes and purification of their antigenic markers are then reviewed, followed by summaries of the extensive literature on tumour cells including an excellent fresh appraisal of the complex "transformation phenotype" by Paster-

nak. Volume 3 closes with three descriptive essays, respectively on the sarcoplasmic reticulum of muscle, composition and structure of excitable nerve membranes and the interactions of sperm and egg membranes in fertilization, and finally a detailed account by De Luca of vitamin A biochemistry and effects on epithelial cell membranes. The role of retinol compounds in transglycosylation is well covered.

To summarise, the series on *Mammalian Cell Membranes* is shaping up as a useful collection of articles which, despite considerable dispersal and overlap between the various volumes and rather indifferent indexing, serves a useful purpose as a source of the recent membrane literature. In one or two cases the author does more, illuminating as well as cataloguing the field. One further point: although the line drawings are in general excellent the reproduction of original photographs and electron micrographs is usually poor. In volume 2, which relies heavily on such reproductions, this is a serious drawback and is always a nuisance since often the pictures illustrate a crucial argument.

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Predator-prey relationships

The Ethology of Predation. By E. Curio. Pp. x+250. (Springer: Berlin and New York, 1976.) DM72; \$29.60.

THIS is a thorough and well-presented review of a wide range of literature up to and including 1973 on the general subject of predator-prey relationships, primarily from a behavioural point of view. It will serve as a valuable reference work for many years to come. Although the main bulk of the book is concerned with behavioural aspects of predation (using the term in a very broad sense), dealing with questions of how animals detect, search for and capture food, there are brief excursions into physiological and ecological aspects of the subject. These peripheral, but important areas are dealt with in less detail than the pure behaviour, and one feels that the author is less happy with them. For example, among the ecological aspects, the discussion of the functional response contains several minor errors, such as the assertion that a type 3 response only occurs when there are at least two alternative prey types. Similarly the summary of optimal foraging theory on pages 70-80, heralded on the book cover as a link

with modern ecology, is confusing and somewhat inaccurate, and the author does not emphasise that the theory he presents at this stage applies equally to problems discussed in other parts of the book. Much of the literature on optimal foraging has appeared since 1973, so Curio's account is already dated.

Curio is, however, encyclopaedic, accurate, and critical in reviewing the more traditional ethological literature. For example, both his discussion of prey selection and the accounts of specialised hunting techniques are excellent sources of information. In the latter sections, the literature survey covers an impressive range of examples, from African natives stalking their prey under the disguise of a stuffed hornbill's head, to Green Herons hunting for fish by dropping pellets of commercial fish food into a pool at the Miami Seaquarium. My only criticism of these behavioural chapters is that the material is presented as a series of rather separate sections without a great deal of attempt to unify them. This makes this useful book more like a work of reference rather than an introduction to the subject.

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Chemisorption on evaporated films

Chemisorption: An Experimental Approach. By G. Wedler. Translated by Derek Klemperer. Pp. vi+250. (Butterworths; London and Boston, Massachusetts, 1976.) £12.

'*Quot homines, tot sententiae*', that venerable Latin tag, is particularly applicable to chemisorption. Since the recent invasion of the subject by physicists and mathematicians it has become so vast, that a full account of the simpler gases on metals alone would require a separate volume for each metal, and a chapter for each lattice plane. Therefore, it is particularly sensible that Professor Wedler, in this broad conspectus of methods, should tend to emphasise the approaches taken by his teacher, R. Suhrmann and himself, to chemisorption on evaporated metal films. This does mean, however, that a proper balance is hardly given to some of the earlier work—for example, Otto Beeck's film work is mentioned but no reference is made to J. K. Roberts' even more crucial studies on tungsten wires, which many regard as a turning point in the subject.

The index reveals no reference to accommodation coefficients or heats of adsorption on wires. Other important more modern techniques are also omitted, such as the Mossbauer effect, and neutron diffraction. The new PhD student starting up in the field would still be well advised to read the Hayward and Trapnell book (*Chemisorption*, Butterworths; London, 1964) to get the scholarly background to

the modern methods described here. But having done this, he or she will find Professor Wedler's book makes easy reading and gives a fairly wide coverage, with 744 references up to 1974. (Here a word of praise should be given to Dr Klemperer for his translation and updating of the German (1970) version.) In addition, it ends up with a short discussion of selected data on Ni/H₂ and Ni/CO, and brings Trapnell's well known Table up to date; it does, however, exclude the hydrocarbons.

Third-year undergraduates doing surface chemistry and research students will find some sections useful, but would be advised to read up nuclear and electron spin resonance elsewhere. How can one possibly explain electron spin resonance in two pages? This is a subject where *g*-factor anisotropies and hyperfine interactions (no mention of these) have contributed vital information covering the adsorbed species on oxides such as SiO₂ and MgO, and a little more effort could have given this topic its proper place in the whole picture.

Experienced workers will want to refer to it for the areas of special expertise of its author, such as electrical and heat measurements on evaporated metal films. Indeed, a better title for the book would surely have been *Chemisorption on Metals*, since there are very few references to metal oxides, or other systems apart from metals. With these qualifications, its purchase can be recommended to science libraries with an interest in the topic concerned.

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Our living planet

Earth, The Living Planet. By Michael J. Bradshaw. Pp. 302. (Hodder and Stoughton; London, Sydney, Auckland and Toronto, 1977.) Paperback £4.95.

THIS volume is intended as "a reader for sixth-form [advanced school] and college courses in physical geography and environmental science", and is a companion to *The Earth's Changing Surface*, about the solid Earth. *Earth, the Living Planet* divides almost equally between a description of the atmosphere, oceans, weather systems and climates of the Earth (the first half of the book) and a description of the kind of life that has evolved and adapted to those conditions. The large (A4) format, generous use of diagrams and a section of colour illustrations help to provide an attractive overall presentation, and make it easy for the reader to dip into the book for selected highlights. In a few places the need for access to the companion volume handicaps the reader who has only bought

this one of the pair; but by and large the book stands well on its own as an introduction to both the physics and the lifeforms of the biosphere.

There are, however, some deficiencies in the presentation, mostly due to over-compression of the material into a limited space. The discussion of weather modification, for example, could well have been developed further, and I was disappointed to find that the chapter "Climates of the Past—and Future?" made no mention of the Milankovitch model of Ice Ages, which in its modern form has now become widely accepted as at least a partial explanation of climatic changes on timescales of a few thousand to several tens of thousand years. By and large, the book provides an excellent introduction to our living planet, at a remarkably reasonable price, and will be useful not only as an introduction for new students but as a refresher for those whose student days are more remote.

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Exploration geophysics

Applied Geophysics. By W. M. Telford, L. P. Geldart, R. E. Sheriff and D. A. Keys. Pp. xvii+860. (Cambridge University; Cambridge, London and New York, 1977.) Hardback £26; paperback £9.75.

THIS textbook of exploration geophysics is intended for practitioners and graduate students, replacing the classic works such as those of Heiland and Jakosky which are now outdated. It stems from another classic, that of Eve and Keyes published twenty years ago, but is far more than a revision of that book, reflecting two decades of particularly rapid advances in both instrumentation and interpretation. The treatment is comprehensive both in coverage of all the important geophysical techniques (developments in remote sensing seem to be the only omission) and in dealing with the practical, theoretical, geological and physical aspects of geophysics in a balanced way. The text is supplemented by problems, mostly using real data, some of which are drawn together in the last chapter to provide examples of integrated interpretation, usually in a mining context.

Over a quarter of the book is concerned with the seismic method. This section has been most successfully updated, and includes sections on the essentials of digital data processing and on the geological interpretation of seismic reflection sections. The refraction method is treated only very briefly but with a clarity which is a feature of the whole book.

The chapters on potential field and electrical methods are rather less satisfactory, giving undue emphasis to instruments, interpretation methods and units now best consigned to the museum: for example, several obsolete methods for the interpretation of resistivity soundings could well have been given as references only, saving space for the 'resistivity transform' approach to this problem. These chapters also assume some knowledge of vector calculus, but the rest of the book should present no mathematical difficulties even to readers trained primarily as geologists.

It is still true that no single textbook is sufficient for the education of a graduate geophysicist, but this one is certainly necessary for reading and reference. At little more than a penny a page in paperback, it has the advantage of being within his means.

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Martian geology

The Geology of Mars. By T. A. Mutch, R. E. Arvidson, J. W. Head, K. L. Jones and R. S. Saunders. Pp. ix+400. (Princeton University: Princeton, New Jersey, 1977.) £28.40; \$35.

WRITING a book about our planetary companions in the Solar System is, and has been for over ten years, a hazardous business. The rate of exploration has been such that inevitably parts of such a book are out-of-date even before it arrives on the bookstands; this is because new, important and often revolutionary information is returned from another exciting space mission. Despite this, and despite the fact that we are in the middle of the highly successful Viking mission to Mars, Thomas Mutch and his colleagues have produced an extremely timely book on the geology of Mars. Essentially, it summarises our knowledge of this planet up to the time of Viking, although it does include pictures and information from the early part of the Viking mission slipped into the book at the last moment. The book is a valuable contribution to planetary studies because it provides (as did Glasstone's *The Book of Mars* before Mariners 6, 7 and 9) an up-to-date summary of our knowledge of this fascinating planet up to the time of Viking—a useful time to stop and take stock of the situation.

The book opens with two chapters describing early telescopic observations and the history of spacecraft exploration of Mars. This is followed in the third chapter by a summary of the major physiographical provinces of Mars and a discussion of how each of the landforms characteristic of different parts of Mars may have been formed. The main bulk of the book consists of individual chapters on surface processes including impact cratering, volcanism and the action of wind and water, all of which have played a part in producing the martian surface as we see it today. There are also discussions of geophysics, structure and geological history of Mars.

Geologists not involved in planetary studies have often been disappointed with new discoveries from both the Moon and Mercury because so much of the geology of these planets is dominated by impact cratering, a geological process of interest to only a few specialists. This is not true of Mars as, although the ancient southern hemisphere of Mars is dominated by impact cratering, the northern hemisphere and poles show abundant

evidence of other processes more familiar to the terrestrial geologist. Large areas have been modified by wind erosion and deposition, and the desert geomorphologist cannot fail to be impressed by the extensive dune fields exposed on the martian surface. Winding channels are indicative of water erosion and the areas around the polar caps show thick sequences of layered rocks. Although volcanism probably played an important part in the evolution of all terrestrial planets, it is only on Mars and Earth that we have seen such a variety of large constructs of high topographical relief. Extensive patterns of rifts and faults show that the tectonic history of Mars has been quite unlike that of the Earth and an understanding of this is essential to developing a general theory for planetary tectonics.

Molecular potential energy surfaces

Symmetry Rules for Chemical Reactions: Orbital Topology and Elementary Processes. By R. G. Pearson. Pp. ix+548. (Wiley-Interscience, New York and London, 1976.) \$31; £17.50.

THE title of this work is not altogether successful in describing the contents. I do not venture to suggest a better one; but Professor Pearson's concern is with the nature of molecular potential energy surfaces generally, and with their influence on the shapes of molecules as well as on the courses of reactions. His thesis rests on two main principles. The first, due principally to Bader, although certainly used earlier, is that a distortion of a particular symmetry is favoured by the presence of a low-lying electronically excited state of a related symmetry. (Perhaps this should be called the 'Bader effect' since the usual 'second-order Jahn-Teller effect' is a clumsy and inaccurate term.) The distortion may lead a molecule from one postulated structure to another, in which case one obtains an understanding of the shapes of molecules. Walsh's rules are usefully approached in this way. Alternatively, the distortion may initiate a reaction, in which case one obtains an understanding of the relative ease of alternative reaction paths.

The second principle is that of "orbital correlation" or "conservation of orbital symmetry": the symmetry species of the molecular orbitals in reactants and products must match. If they do not, then at least one bonding orbital in the reactants correlates with an anti-bonding orbital in the products,

The authors of this book do an excellent job of summarising and discussing critically our knowledge of all these subjects. They also briefly compare Mars with the other planets, including Earth, showing how all the solid planets in the Solar System are important field areas for Earth-bound geologists.

This book will not only be a useful reference book for planetary geologists but provides an easily readable and well illustrated review for the non-specialist. For a student it is an important text, although sadly the price is high (especially the sterling equivalent); and students may have to rely on library copies. **J. E. Guest**

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which in turn implies a high activation energy. In the application of these principles, a third is used: the predicted behaviour depends on the "orbital topology"—the nodal structure of the molecular orbitals—which is not significantly modified even if the symmetry is broken by irrelevant changes to the molecule. Consequently symmetry-based methods may often be used where no formal symmetry is present.

Professor Pearson expounds these principles with admirable clarity and numerous examples. The Jahn-Teller selection rule is incorrectly stated (the correct version is subsequently used, but with no explanation of the notation for the symmetrised square) and the account of the first-order Jahn-Teller effect contains some very dubious remarks. Fortunately, the first-order Jahn-Teller effect is of only peripheral importance in the later development. A more general criticism is that the magnitudes of the effects cited are not always shown to be sufficient to account quantitatively for the observations. For example, the dissociation energy of ozone is 1 eV, whereas that of sulphur dioxide is 5.5 eV; this is explained by the fact that the relevant excited state in ozone is at 4.7 eV, whereas the corresponding state in sulphur dioxide is at 5.3 eV. The difference is in the right direction but the numerical values are unconvincing.

For the most part, however, Professor Pearson approaches his subject carefully, critically and thoroughly. This is a stimulating and valuable book, and an important contribution to a rapidly developing field of research.

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